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CAMBRIDGE UNIVERSITY PRESS
LONDON: BENTLEY HOUSE, N.W.1
NEW YORK: 32 EAST 57TH STREET, 22

Price 20s. net

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The Occurrence of *n*-Heptadecanoic Acid (Margaric Acid) in Hydrogenated Mutton Fat

By R. P. HANSEN, F. B. SHORLAND and N. JUNE COOKE
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(*Received* 28 *June* 1954)

Although the isolation of *n*-heptadecanoic acid (margaric acid) from natural fats has often been reported (cf. Ralston, 1948), there appears to be no case on record where the identification of the acid from these sources has been irrefutably established. Ralston (1948) states: 'All the naturally occurring heptadecanoic acids which have been described and subsequently investigated have been shown to consist of mixtures of palmitic and stearic acids. Because of the frequent mutual occurrence of palmitic and stearic acids in fats, and since they are often the chief saturated acids, it is not surprising that a mixture of them has frequently been identified as a pure compound.' Not only has the presence of margaric acid in fats been unsubstantiated, but so also has the presence of the other straight-chain odd-numbered fatty acids with the exception of the recently reported steam-volatile acids of ox perinephric fat, which were shown to contain trace amounts of all the straight-chain acids (both odd and even) from C_2 to C_{10} (Hansen & McInnes, 1954). This latter work has prompted further investigations into the minor components of fats, and as a preliminary to a comprehensive search for odd-numbered straight-chain acids the 'solids' from a sample of hydrogenated mutton fat (external carcass fat) have been investigated and have yielded margaric acid. Brief reference to this finding has already been made (Hansen, Shorland & Cooke, 1954). From the same original material used in this investigation and by similar techniques which included hydrogenation, fractional distillation and low-temperature crystallization, were isolated small quantities of three branched-chain fatty acids, namely, 12-methyltetradecanoic acid, 13-methyltetradecanoic acid (Hansen, Shorland & Cooke, 1953) and 14-methylhexadecanoic acid (Hansen, Shorland & Cooke, 1952).

EXPERIMENTAL

The fat used in this investigation (Lab. no. G/48, sap. equiv. 286·9, iodine value 46·6, unsaponifiable matter 0·56% and free fatty acids 0·4%) was obtained, as described earlier (Hansen *et al.* 1952, 1953), by mincing and steam-rendering the external fatty tissues from the carcasses of old overweight ewes. The washed glycerides were converted into methyl esters (7·69 kg.) and hydrogenated at 180°, using as catalyst N; supported on kieselguhr. The hydrogenated methyl esters (iodine value 3·0) were then repeatedly crystallized at −30° from 10 vol. of acetone. Of the resulting acetone-insoluble methyl esters ('solids'), 6·45 kg. were fractionated *in vacuo* in a 490 × 3·8 cm. stainless-steel column packed with 3–4 mm. diameter single-turn glass helices. Sixteen fractions and a large residue resulted. For the purpose of this paper the tenth fraction (denoted 0S10, 232·8 g.; sap. equiv. 281·5, iodine value 0·8) was selected for further investigation. A portion of fraction 0S10 (denoted 01, 22·0 g.) was then refractionated at approximately 0·1 mm. pressure in a 50 × 1·8 cm. column fitted with a closely coiled spring as packing (column *E*, Shorland, 1952). Fractionation data are shown in Table 1.

Table 1. *Fractional distillation of methyl esters* (01)

Wt. 22·0 g.; sap. equiv. 281·5; iodine value 0·7. Pressure approx. 0·1 mm.

Fraction	Wt. (g.)	Sap. equiv.	M.p. of ester (°)	M.p. of acid (°)
01S1	0·75	279·3	27·5–28·9	—
01S2	1·73	271·4	29·4–29·8	—
01S3	0·99	268·2	29·5–30·0	—
01S4	1·75	273·4	23·9–27·0	—
01S5	0·91	285·0	20·5–21·7	—
01S6	1·48	283·8	21·0–23·9	53·0–54·0
01S7	0·70	283·5	24·5–24·7	
01S8	3·12	284·1	25·5–26·6	57·0–57·8
01S9	1·20	284·0	27·0–29·6	60·3–60·7
01S10	1·45	282·5	29·8–30·5	60·8–61·0
01S11	0·75	282·8	28·2–30·6	60·6–60·7
01S12	2·42	284·7	29·6–30·2	
01S13	1·73	288·7	28·1–29·5	—
01S14	0·55	288·9	31·6–33·3	—
01SR	2·59	319·5	—	—

Those fractions with saponification equivalents approximating 284 were then examined in detail. Typical of the procedure adopted was the case of methyl ester fraction 01S9 (1·20 g.; sap. equiv. 284·0, m.p. 27·0–29·6°), which was converted into fatty acids (denoted 02, 1·03 g.; m.p. 60·3–60·7°) and submitted to low-temperature crystallization from light petroleum (b.p. 50–60°), methanol, acetone and ether respectively, to yield fraction 02S3LSS. The chemical and physical properties of fraction 02S3LSS (0·85 g.) were as follows: m.p. 61·3–61·4°; sap. equiv. 270·2; X-ray long spacing 40·8 Å; combustion analysis, C, 75·5; H, 12·5%; iodine value 0·0; n_D^{63} 1·4312 (a pure sample of *n*-heptadecanoic acid m.p. 61·1–61·2° under identical conditions gave n_D^{63} 1·4312). Fraction 02S3LSS, when mixed in equal proportions with pure *n*-heptadecanoic acid (m.p. 61·1–61·2°), gave mixed m.p. 61·0–61·3°. By similar processes of low-temperature crystallization the following acid fractions were obtained: 08S9S (derived from bulked fractions 01S6 and 01S7 acids (08)) 0·65 g.; m,p. 61·0–61·3°, sap. equiv. 269·0. 03S8S (derived from 01S8) 1·77 g.; m.p. 61·1–61·3°. 04S3S (derived from 01S10) 1·05 g.; m.p. 61·0–61·3°. 05SS (derived from bulked fractions 01S11 and 01S12 acids (05)) 1·66 g.; m.p. 61·2–61·6°.

The above fractions (08S9S, 03S8S, 04S3S and 05SS) were bulked together (denoted 010) and further purified by low-temperature crystallization to yield 010S3S (4·54 g.) with the following properties: m.p. 61·3–61·6°, sap. equiv. 270·3; C, 75·8; H, 12·6%; iodine value 0·0; X-ray long spacing 40·5 Å; n_D^{63} 1·4310.

The X-ray measurements reported in this paper were made with a Philips Geiger X-ray spectrometer using nickel-filtered Cu *K*α radiation for fraction 02S3LSS and manganese-filtered Fe *K*α radiation for fraction 010S3S.

Melting points were determined in closed capillaries and are uncorrected.

Combustion analyses were made by Drs G. Weiler and F. B. Strauss, Oxford.

DISCUSSION

The two fractions reported in this paper 02S3LSS and 010S3S possess saponification equivalents (270·2 and 270·3) carbon and hydrogen contents (C, 75·5; H, 12·5%; and C, 75·8; H, 12·6%) and iodine values (0·0 and 0·0) which signify a C_{17} satured fatty acid. (Calculated requirements for a saturated fatty acid with empirical formula $C_{17}H_{34}O_2$: sap. equiv. 270·4; C, 75·5; H, 12·7%; iodine value, 0·0.) The X-ray long spacing of each of the fractions (40·8 Å and 40·5 Å) agrees within the experimental error (±0·5 Å) with the values of 40·45 Å (Francis & Piper, 1939) and 40·05 Å (Slagle & Ott, 1933) for *n*-heptadecanoic acid. These X-ray diffraction measurements show the two fractions to be almost identical and at the same time distinct from two of the methyl-branched isomers, 15-methylhexadecanoic acid and 14-methylhexadecanoic acid, which have long spacings of 33·35 Å (Arosenius, Ställberg, Stenhagen & Tägström-Eketorp, 1949) and 29·9 Å (Nunn, 1952) respectively. The melting points of the two fractions (61·3–61·4° and 61·3–61·6°) are in close agreement with the

Table 2. *Purification of fatty acids by fractional crystallization of fraction 02*

For details see text.

			Soluble			Insoluble		
Fraction	Wt. (g.)	Conditions of crystallization	Fraction	Wt. (g.)	M.p. (°)	Fraction	Wt. (g.)	M.p. (°)
02	1·03	Light petroleum (40 vol.), −40°	02L	0·03	31·0–31·2	02S	1·00	61·0–61·3
02S	1·00	Methanol (40 vol.), −40°	02SL	0·02	46·0	02SS	0·99	61·1
02SS	0·99	Acetone (40 vol.), −40°	02S2L	0·02	37·3	02S2S	0·97	61·1–61·2
02S2S	0·97	Light petroleum (100 vol.), +18°	02S3L	0·96	61·1–61·3	02S3S	0·01	59·6–60·1
02S3L	0·96	Ether (40 vol.), −40°	02S3LL	0·05	56·8–58·2	02S3LS	0·91	61·2–61·4
02S3LS	0·91	Ether (40 vol.), −40°	02S3LSL	0·05	59·8–60·3	02S3LSS (Saponification equiv. 270·2)	0·85	61·3–61·4

recorded figures for *n*-heptadecanoic acid (61·3°, Francis & Piper, 1939; 59·0–60·0°, Levene & West, 1914). Fraction 02S3LSS when mixed in equal proportions with pure margaric acid (m.p. 61·1–61·2°) did not depress the melting point. Evidence which precludes these two fractions consisting of equimolecular mixtures of palmitic and stearic acids is furnished by the data of Schuette & Vogel (1940), Smith (1936) and Francis, Collins & Piper (1937), which indicate a maximum solidification point of about 56° for such a mixture. Margaric acid is reported by Waterman & Bertram (1927) to have a refractive index of n_D^{60} 1·4319, and this value is similar to that obtained for fractions 02S3LSS (n_D^{63} 1·4312) and 010S3S (n_D^{60} 1·4310). The chemical and physical characteristics of 02S3LSS and 010S3S as recorded above establish the two fractions isolated from hydrogenated mutton fat as being the C_{17} straight-chain fatty acid *n*-heptadecanoic acid.

It is estimated that *n*-heptadecanoic acid constitutes about 1·2% of the total fatty acids of hydrogenated mutton fat. The two fractions 02S3LSS and 010S3S (5·39 g.) were separated from a 22·0 g. portion of the original fraction 0S10 (232·8 g.), and this proportion represents a total weight of 57·1 g. of pure margaric acid in the original fraction.

As the methyl esters in the present investigation had been submitted to hydrogenation, it has not been determined whether margaric acid exists as such (in part or in whole), or as a C_{17} unsaturated acid, in the unhydrogenated fat. The possibility that the heptadecanoic acid now isolated could have originated from a C_{17} acid containing a cyclopropane ring is also not excluded. Hofmann & Lucas (1950) and Hofmann, Lucas & Sax (1952) have shown that there occurs in the non-glyceridic lipids of *Lactobaccilus arabinosus* a C_{19} saturated acid with a cyclopropane ring which is opened on hydrogenation to yield *n*-nonodecanoic acid and a methyloctadecanoic acid.

Whatever may be the chemical structure of the C_{17} acids in the original external carcass fat of sheep, it is probable that corresponding acids exist in other natural glycerides also. Furthermore, it is reasonable to presume that not only C_{17} acids are present, but also all odd-numbered members in the range characteristic of the fat. In order to elucidate this problem, work on the nature of C_{17} and other odd-numbered straight-chain fatty acids is already in progress in this laboratory.

SUMMARY

Hydrogenated mutton fat has been found to contain approximately 1·2% of *n*-heptadecanoic acid.

We wish to thank Mr M. Fieldes, Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand, for the X-ray measurements reported in this paper.

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The Occurrence of *n*-Pentadecanoic Acid in Hydrogenated Mutton Fat

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(*Received* 19 *July* 1954)

When *n*-heptadecanoic acid (margaric acid) was isolated from hydrogenated mutton fat (Hansen, Shorland & Cooke, 1954*a*) it was anticipated that other fatty acids of high molecular weight and with an odd number of carbon atoms would also be present in this material. Further investigations have now been made and *n*-pentadecanoic acid has been found in the same hydrogenated sample of external carcass fat from old ewes.

n-Pentadecanoic acid has not hitherto been established as a component of natural fats, although unpublished evidence obtained by Morice & Shorland in this laboratory indicates that it is present in hydrogenated shark-liver oil. It has, however, been found (together with other fatty acids containing an odd number of carbon atoms) in synthetic fats manufactured from 'gatsch' (cf. Williams, 1947).

The sample of mutton fat used in this investigation was earlier shown to contain small quantities of *n*-decanoic acid (capric acid; Hansen & Cooke, 1953) together with the branched-chain acids 12-methyltetradecanoic acid, 13-methyltetradecanoic acid (Hansen, Shorland & Cooke, 1953), and 14-methylhexadecanoic acid (Hansen, Shorland & Cooke, 1952). *n*-Pentadecanoic acid is thus the third C_{15} acid to be identified from this source.

EXPERIMENTAL

As formerly reported (Hansen *et al.* 1952, 1953, 1954*a*; Hansen & Cooke, 1953) the fat used in this work (sap. equiv. 286·9, iodine val. 46·6, unsaponifiable matter 0·56%, and free fatty acids 0·4%) was extracted by steam-rendering the minced external fatty tissues cut from the carcasses of old overweight ewes. The glycerides were converted into methyl esters (7·69 kg.), which were hydrogenated at 180° using a Ni catalyst supported on kieselguhr, and then repeatedly crystallized from 10 vol. acetone at −30°. Fractional distillation of 6·45 kg. of the acetone-insoluble esters ('solids') was then carried out in a 490 × 3·8 cm. stainless-steel column packed with 3–4 mm. diameter single-turn glass helices. Of the 16 fractions and a large residue which resulted, the third (denoted OS3, wt. 40·0 g., sap. equiv. 253·6, iodine val. 0·5) was selected for this investigation. Part of fraction OS3 (34·76 g.) was refractionated *in vacuo* (0·1–0·5 mm.) in a 50 × 1·8 cm. column (column *E*, Shorland, 1952) to yield ten fractions (OS3S1 to OS3S10) and a residue as shown in Table 1.

Fractions OS3S6 (denoted O15, wt. 4·30 g., sap. equiv. 256·5, m.p. 17·8–18·2°; m.p. (acids) 49·6–50·4°) possessed characteristics similar to those of methyl pentadecanoate. Accordingly the acids of O15 were submitted to low-temperature crystallization from 40 vol. of the following solvents at −40°, the soluble fraction being removed after each crystallization: light petroleum (b.p. 50–60°) (3 crystallizations); methanol (6 crystallizations); ether (1 crystallization); and acetone (2 crystallizations). The resulting purified acid fraction O15S11S (wt. 2·39 g.) had the following physical and chemical properties: m.p. 52·8–53·1°; sap. equiv. 242·4; combustion analyses: C, 74·5; H, 12·5%; iodine val. 0·0; X-ray long spacing 35·5 Å; n_D^{80} 1·4328: methyl ester m.p. 18·4–19·0°; methyl ester n_D^{20} 1·4402. The acid fraction when mixed in equal proportions: (*a*) with the C_{15} *iso* acid from butterfat (K14S17LS3S, m.p. 52·0°, Hansen, Shorland & Cooke, 1954*b*) gave a mixed m.p. of 45·5–46·0°, (*b*) with the C_{15} *iso* acid synthesized by Arosenius, Ställberg, Stenhagen & Tägtström-Eketorp (1949), m.p. 51·7–51·8°, it gave a mixed m.p. of 44·5–45·2°.

Table 1. *Fractional distillation of methyl esters* (OS3)

Wt. 34·76 g., sap. equiv. 253·6, iodine val. 0·5.

Fraction	Wt. (g.)	M.p. (°)	Saponification equiv.	Designation for further work
OS3S1	3·33	18·7–19·2	244·7	—
OS3S2	2·73	18·5–18·7	242·8	—
OS3S3	3·74	17·2–17·5	244·2	—
OS3S4	3·89	8·8– 9·2	251·3	O17
OS3S5	4·54	13·0–13·8	256·6	
OS3S6	4·30	17·8–18·2	256·5	O15
OS3S7	1·37	16·4–17·5	256·7	O16
OS3S8	4·02	17·4–18·1	256·7	
OS3S9	2·37	22·9–23·8	264·6	O18
OS3S10	1·29	26·8–29·1	268·0	—
OS3SR	2·85	—	280·2	—

By similar processes of low-temperature crystallization of the fatty acids, other fractions reported in Table 1, namely OS3S4 and OS3S5 (combined and denoted O17), OS3S7 and OS3S8 (combined and denoted O16), and OS3S9 (denoted O18), yielded varying amounts of purified acid. The largest of these fractions was O16S7S (wt. 4·19 g.) which possessed the following characteristics: m.p. 52·7–53·2°; sap. equiv. 242·3; combustion analyses: C, 74·6; H, 12·7%; iodine val. 0·0; X-ray long spacing 36·1 Å; n_D^{60} 1·4328.

The X-ray measurements reported in this paper were made with a Philips Geiger X-ray spectrometer using manganese-filtered Fe $K\alpha$ radiation. Combustion analyses were made by Drs G. Weiler and F. B. Strauss, Oxford. Melting points were determined in closed capillaries and are uncorrected.

DISCUSSION

A study of the following chemical and physical properties of fractions O15S11S and O16S7S respectively, establishes the presence in hydrogenated mutton fat of the C_{15} saturated straight-chain fatty acid *n*-pentadecanoic acid: saponification equivalents 242·4 and 242·3 (calc. for $C_{15}H_{30}O_2$: 242·4); combustion analyses: C, 74·5; H, 12·5 and C, 74·6; H, 12·7% (calc. for $C_{15}H_{30}O_2$: C, 74·4, H, 12·5%); iodine values 0·0 for O15S11S and O16S7S; m.p.'s 52·8–53·1° and 52·7–53·2°; values from the literature are as follows: Francis, Piper & Malkin (1930) 52·1°; Levene & West (1914) 53·0°; Links & de Groot (1953) 52·3–53·3°; Coops quoted by Links & de Groot 54·4°; Weitkamp (1945) 52·4°; Meyer & Reid (1933) 52·26°. X-ray long spacing 35·5 and 36·1 Å (Francis *et al.* (1930) 35·8 Å; Slagle & Ott (1933) 35·75 Å); refractive index for both fractions n_D^{60} 1·4328 (Dorinson, McCorkle & Ralston (1942), n_D^{60} 1·4329).

In his paper on the acidic constituents of degras, Weitkamp (1945) stated that all the *iso* acids with an even number of carbon atoms, when mixed with their corresponding normal acids, showed a depression of 10–15° in melting point. Investigations made in this laboratory indicate that in the case of *iso* acids with an odd number of carbons the depression appears to be smaller. The normal C_{15} acid reported in this paper (m.p. 52·8–53·1°) when mixed in equal proportions with the C_{15} *iso* acid from butterfat (K14S17LS3S, m.p. 52·0°, Hansen *et al.* 1954*b*) gave a mixed melting point of 45·5–46·0° (depression, 7–8°). Similarly, our normal C_{15} acid when admixed with the C_{15} *iso* 13-methyltetradecanoic acid (m.p. 51·7–51·8°) kindly supplied by Professor E. Stenhagen (Arosenius *et al.* 1949) gave a mixed melting point of 44·5–45·2° (depression, 7–8°).

In the sample of hydrogenated mutton fat examined, *n*-pentadecanoic acid is present to the extent of approximately 0·15% of the total fatty acids.

SUMMARY

Hydrogenated mutton fat has been found to contain approximately 0·15% of *n*-pentadecanoic acid.

We wish to acknowledge the assistance given by Mr M. Fieldes, Soil Bureau, Department of Scientific and Industrial Research, Wellington, who made the X-ray measurements reported in this paper.

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A Note on the Chromatographic Identification of Blood Keto Acids in Animals poisoned with Arsenite and Alloxan

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(*Received* 29 *May* 1954)

In agreement with the original observations of Cavallini, Frontali & Toschi (1949) on the separation of keto acid 2:4-dinitrophenylhydrazones by paper chromatography, El Hawary & Thompson (1953) demonstrated that when pure pyruvic acid 2:4-dinitrophenylhydrazone is applied to filter paper and chromatographed with a butanol–ethanol–ammonia–water mixture, a single spot, having R_F about 0·4, is obtained. With the samples of pyruvic acid and its 2:4-dinitrophenylhydrazone which we were then using we found no evidence at that time of the presence of a second spot due to pyruvic acid 2:4-dinitrophenylhydrazone, even when the pyruvic acid derivative was taken through the whole extraction process which we were using for the separation of 'hydrazones' from blood.

When the keto acid hydrazones extracted from human blood were separated chromatographically, however, we found, in addition to the α-oxoglutarate and pyruvate derivatives, a third spot having R_F slightly higher than that for the pyruvate hydrazone. We had further shown that the acetoacetic acid hydrazone, when applied directly to paper, has under our conditions, R_F similar to that for the third blood spot, although when it is carried through the extraction process it is partially decarboxylated to the acetone hydrazone, having R_F 0·90–0·95; this latter finding has been confirmed by Markees (1954). We therefore provisionally identified the third spot in the blood chromatograms as due to acetoacetic acid, although we stated that we had been unable to get good agreement between the absorption curve of the pure acetoacetic acid hydrazone and that of the solution eluted from the third blood spot. We suggested that this lack of agreement might have been due to the blood spot containing traces of the acetone hydrazone formed by decarboxylation of the acetoacetic acid derivative while running on the paper.

Several workers, however, have reported the presence of two spots in chromatograms of the hydrazone derived from pure pyruvic acid (LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953; Stewart, 1953). In further work of our own we have confirmed this finding, using pure sodium pyruvate or freshly distilled pyruvic acid and extracting the hydrazone formed from this by the usual procedure used for the extraction of hydrazones from blood. However, when pyruvic acid 2:4-dinitrophenylhydrazone is applied directly to the paper, without preliminary extraction, we still regularly obtain one spot. When the hydrazone is taken through the extraction process, the relative intensity of the second spot varies widely under our conditions, corresponding in some cases to less than 5% of the total pyruvic acid, and in others to more than 50%.

It has further been stated that the third spot usually visible in chromatograms of blood filtrates has R_F and absorption curve similar to those for the more rapidly moving spot produced by the pyruvic acid hydrazone (Cavallini & Frontali, 1954). Kulonen, Carpén & Ruokolainen (1952), however, had earlier claimed that the R_F value for this faster pyruvic acid component corresponds to that for the acetoacetic acid hydrazone, although Markees (1954) has stated that under his conditions the R_F of the latter is intermediate between the values for the two pyruvate components.

Because of these conflicting findings we have studied chromatographically the blood of animals poisoned with sodium arsenite or alloxan, and containing therefore high levels of pyruvic acid (Peters, Sinclair & Thompson, 1946) and acetoacetic acid (Lackey, Bunde, Gill & Harris, 1944) respectively. By this means it was hoped to provide further evidence towards the identification of the blood constituent forming this third spot. These experiments form part of a more comprehensive study of the actions *in vivo* of a number of enzyme inhibitors on the level of various blood constituents, which will be reported later together with a fuller account of the physiological state of these poisoned animals.

EXPERIMENTAL

Chromatographic procedure. The preparation and extraction of keto acid 2:4-dinitrophenylhydrazones from blood, and the chromatographic procedure and subsequent estimation of the separated hydrazones, were carried out as described earlier (El Hawary & Thompson, 1953).

Arsenite-poisoned animals. A solution of sodium arsenite, prepared by dissolving As_2O_3 in 0·1 N-NaOH, was neutralized

and injected subcutaneously into rats in 0·2 ml. doses equivalent to 12 mg. As_2O_3/kg. body wt. The animals were killed by decapitation 2 hr. later, and blood was drained directly into the appropriate protein precipitant. The amount of blood taken was then determined by reweighing the tubes.

Alloxan-poisoned animals. 200 mg. alloxan/kg. body wt. was injected intraperitoneally into rats. The animals were killed after hyperglycaemia had developed, i.e. 48–72 hr. later, and blood samples obtained as described above.

Acetoacetic acid was estimated in blood by a modification of the method of Thin & Robertson (1952). We have used 10% (w/v) trichloroacetic acid as a protein precipitant instead of the $Ba(OH)_2$ and $ZnSO_4$, $7H_2O$ used by these workers. We have also modified the colour reagent, using 1 ml. of a mixture of 0·5 ml. salicylic aldehyde with 8 ml. 4N-KOH. After incubation for 2 hr. at 37°, we have removed the 1 ml. from the centre well of the Conway units with a Pasteur pipette and have then washed out the well twice with 1 ml. of distilled water.

RESULTS

Chromatograms were prepared from the keto acid 2:4-dinitrophenylhydrazones extracted from the blood of seven normal rats, seven poisoned with arsenite and five with alloxan. The pyruvate spot (i.e. with R_F corresponding to that of the single spot obtained when the pure pyruvic acid 2:4-dinitrophenylhydrazone is applied to the paper) and the 'third spot' present in the blood filtrates (i.e. with R_F resembling both that of acetoacetate and that of the faster component derivable from pyruvic acid) were cut out from the papers and the hydrazone eluted and estimated colorimetrically. Further blood samples were taken from each animal for estimation of acetoacetate. The results are shown in Table 1.

In confirmation of Peters *et al.* (1946) it will be seen that sodium arsenite, administered in these doses, causes a fivefold increase in the blood pyruvate level; the level of acetoacetate however is only 1·8 times that in the normal animals. With alloxan, on the other hand, the level of acetoacetate is increased ninefold, under our conditions while that of pyruvate is only 1·9 times the normal level.

Furthermore, the rise in the pyruvate level in the arsenite-poisoned rats is accompanied by an increase in the third spot, whereas the marked rise in the acetoacetate level in the alloxan animals is associated with a fall in the mean level of the substance responsible for the third spot.

The level of α-oxoglutarate is also increased in both the arsenite- and alloxan-poisoned groups, but it is not proposed to describe in detail results obtained for this keto acid since it yields only one spot under our conditions (see also Cavallini & Frontali, 1954) and is widely separated from the pyruvate and acetoacetate spots.

DISCUSSION

Although it is now known that pure sodium pyruvate gives rise to two dinitrophenylhydrazone components having different R_F values, the presence of both pyruvate and acetoacetate in blood, together with the conflicting statements concerning the R_F values of the dinitrophenylhydrazones derived from these substances, made it desirable to determine with more certainty which of these two acids is responsible for the third component normally visible in chromatograms of blood extracts, or whether both acids contribute to variable degrees.

It is clear from the results described above that, in our earlier provisional identification of this third spot as due to acetoacetate (El Hawary & Thompson, 1953), we were misled by the close similarity under our conditions between the R_F values of the acetoacetate dinitrophenylhydrazone and of the faster pyruvate component. Despite the wide variations in the relative intensity of the second pyruvate spot which we have found in different experiments with pure sodium pyruvate, the present work supports the conclusion of Markees (1954) that this third component in chromatograms of blood extracts is due to pyruvate, since even when the

Table 1. *Blood keto acid levels in normal rats and in rats poisoned with sodium arsenite and alloxan*

Expressed as mg. acid/100 g. blood: 'third spot' expressed in terms of pyruvic acid.

	Pyruvate	'Third spot'	Aceto-acetate
Normal	0·52	0·38	0·40
	0·42	0·38	0·74
	0·51	0·35	0·62
	0·33	0·37	0·38
	0·46	0·19	0·72
	0·56	0·27	0·53
	0·65	0·08	0·30
Mean ± S.E.M.	0·49 ± 0·04	0·29 ± 0·04	0·53 ± 0·07
Arsenite-poisoned	1·70	0·86	0·55
	1·15	1·06	0·67
	1·78	0·64	0·90
	3·22	0·31	0·95
	4·23	1·26	1·30
	2·73	1·27	1·07
	2·05	0·42	1·06
Mean ± S.E.M.	2·46 ± 0·37	0·83 ± 0·15	0·93 ± 0·10
Alloxan-poisoned	1·00	0·35	5·10
	0·63	0·39	4·75
	0·97	0·06	5·95
	0·85	0·04	4·05
	1·32	0·05	4·22
Mean ± S.E.M.	0·95 ± 0·11	0·18 ± 0·08	4·81 ± 0·34
Ratio of means			
$\frac{\text{Arsenite-poisoned}}{\text{Normal}}$	5·0	2·9	1·8
$\frac{\text{Alloxan-poisoned}}{\text{Normal}}$	1·9	0·6	9·1

acetoacetate level is markedly raised it does not contribute to any increase in the third spot. The absence of any detectable acetoacetate spot is probably due to decarboxylation, both during the extraction process and during the run on the paper, and it was noticed that an acetone 2:4-dinitrophenylhydrazone spot was regularly seen on the chromatograms from the alloxan-treated animals.

In view of these findings, together with the resemblance between the absorption curve of the substance giving the third spot in blood filtrates with that of the second pyruvate spot (Cavallini & Frontali, 1954), which finding we have also confirmed, we conclude that this third blood component does not result from acetoacetic acid but corresponds to the more rapidly moving pyruvate component. It would seem therefore that a safe estimate of the pyruvate content of blood can be obtained by summing the two more rapidly moving spots, even when high levels of acetoacetate are present.

SUMMARY

1. A chromatographic study has been made of the keto acids present in the blood of normal rats and of rats poisoned with sodium arsenite and alloxan respectively.

2. It has been shown that a rise in the blood pyruvate level is accompanied by a rise in the 'third spot' visible in chromatograms of dinitrophenylhydrazones from blood, and that no increase in this occurs when the acetoacetate level is high.

3. It is concluded that this third spot is due to the more rapidly running component of the pyruvic acid 2:4-dinitrophenylhydrazone, and that high acetoacetate levels are not likely to interfere in the chromatographic estimation of the blood pyruvate level.

Our thanks are due to Mr P. G. Hunt for his skilled technical assistance.

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Analogues of Diaminopimelic Acid as Inhibitors of Bacterial Growth

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(*Received* 14 *May* 1954)

Many analogues of amino acids have been synthesized and tested as potential bactericides. β-2-Thienylalanine inhibits growth of *Escherichia coli*, *Streptococcus faecalis* and *Lactobacillus arabinosus*, but it has no chemotherapeutic value, since it is also toxic to animals (du Vigneaud, McKennis, Simmonds, Dittmer & Brown, 1945; Dittmer, Ellis, McKennis & du Vigneaud, 1946; Ferger & du Vigneaud, 1948). This, and much other work, has made it evident that chemical analogues of the amino acids which are normal constituents of animal proteins are unlikely to display sufficient selective toxicity to make them valuable chemotherapeutic drugs.

The demonstration (Work, 1951; Work & Dewey, 1952, 1953) that αα′-diaminopimelic acid was confined to the bacterial kingdom, suggested that analogues of this compound might inhibit bacterial metabolism without being toxic to animal cells. The present communication records the synthesis of a series of such analogues and their derivatives, together with tests on these compounds as inhibitors of bacterial growth. Their behaviour as competitive inhibitors for the specific diaminopimelic acid decarboxylase of bacterial cells is recorded in the following paper (Dewey, Hoare & Work, 1954).

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SYNTHETIC WORK

αα′-Diaminoadipic acid. This was prepared by condensation of dimethyl αα′-dibromoadipate (Guha & Sankaran, 1946), with potassium phthalimide in dimethylformamide, followed by hydrolysis of the resulting dimethyl αα′-diphthalimidoadipate with hydrazine hydrate and 12N-HCl (Sheehan & Bolhofer, 1950). After the filtrate had been neutralized with 2N-NH_3, the product crystallized from water and melted at 286–298° with decomposition. (Found: C, 41·1; H, 6·7; N, 15·7. Calc. for $C_6H_{12}O_4N_2$: C, 40·9; H, 6·8; N, 15·9%.)

αα′-Diaminopimelic acid. This was prepared as above by condensation of diethyl αα′-dibromopimelate with potassium phthalimide to yield diethyl αα′-diphthalimidopimelate as a syrup. This was hydrolysed with hydrazine hydrate and 12N-HCl. The product did not precipitate after neutralization of the filtered hydrolysate with 3N-NH_3. Ammonium chloride was therefore removed in an electrodialysis cell, and αα′-diaminopimelic acid precipitated with ethanol from the solution after concentration (yield 30%). It charred at 312° and decomposed at 345–351°. (Found: C, 43·8; H, 7·4; N, 14·9. Calc. for $C_7H_{14}O_4N_2$: C, 44·2; H, 7·4; N, 14·8%.)

αα′-Diaminosuberic acid. The tetraethyl ester of 1:6-*diacetamidohexane*-1:1:6:6-*tetracarboxylic acid* was prepared by refluxing 1·3 moles of redistilled tetramethylene dibromide with 2 moles of ethyl acetamidomalonate and 2 g. atoms of Na in dry ethanol for 6 hr. Yield, 52% on ethyl acetamidomalonate; from ethylacetate–light petroleum (b.p. 60–80°), m.p. 136–137°. (Found: C, 54·2; H, 7·6; N, 5·8. $C_{22}H_{36}O_{10}N_2$ requires C, 54·0; H, 7·4; N, 5·7%)

Hydrolysis of this material was carried out with 48% (w/w) aqueous HBr at 160° for 7 hr. The reaction mixture was evaporated to remove HBr and the dry residue was taken up in ethanol. Excess 50% (v/v) ethanolic pyridine was added to precipitate the αα′-diaminosuberic acid (85%). The product darkened slightly at 280–360° but did not melt below 360° (cf. Abderhalden & Zeisset, 1928). (Found: C, 47·0; H, 7·6; N, 13·7. Calc. for $C_8H_{16}O_4N_2$: C, 47·2; H, 7·8; N, 13·8%.)

αα′-Diaminoazelaic acid. This was prepared by amination of αα′-dibromoazelaic acid with $(NH_4)_2CO_3$ and aqueous NH_3 (sp.gr. 0·880). The product (84% yield) charred at 310° but did not melt below 360° (Neuberg & Niemann, 1905). (Found: C, 48·9; H, 8·4; N, 11·4. Calc. for $C_9H_{18}O_4N_2$: C, 49·5; H, 8·3; N, 12·9%.)

It was also prepared by condensation of ethyl acetamidomalonate (2 moles) with pentamethylene dibromide (1·3 moles) in the presence of sodium ethoxide (2 moles) as above to yield the *tetraethyl ester of* 1:7-*diacetamidoheptane*-1:1:7:7-*tetracarboxylic acid* (42% on acetamidomalonic ester), m.p. 148·5–149·5°. (Found: C, 55·4; H, 7·5; N, 5·8. $C_{23}H_{38}O_{10}N_2$ requires C, 54·9; H, 7·6; N, 5·6%.) This was hydrolysed with 48% HBr (12 moles) as described for αα′-diaminosuberic acid. αα′-Diaminoazelaic acid (92% yield) did not melt below 360°. (Found: C, 48·9; H, 8·0; N, 12·6. Calc. for $C_9H_{18}O_4N_2$: C, 49·5; H, 8·3; N, 12·9%.)

αα′-Diaminosebacic acid. Prepared by the bromination of sebacic acid and amination of the resulting αα′-dibromosebacic acid (Neuberg & Niemann, 1905). Free αα′-diaminosebacic acid was prepared from its hydrochloride by precipitation with 2N-NH_3. It charred at 330–340° but did not melt below 360° (yield 85%.) (Found: C, 49·7; H, 8·7; N, 11·9. Calc. for $C_{10}H_{20}O_4N_2$: C, 51·7; H, 8·6; N, 12·1%.)

αα′-Diaminosebacic acid diamide. Diethyl αα′-dibromosebacate was treated with $(NH_4)_2CO_3$ and liquid NH_3 in a sealed tube at room temperature for 1 week. The NH_3 was allowed to evaporate spontaneously, and recrystallization of the amide was attempted from water and ethanol, in both of which it was exceedingly soluble. The highest m.p. recorded (142–147° with decomposition) was obtained with a sample which had been recrystallized from ethanol. Analytical figures were unsatisfactory.

αα′-Bis(dimethylamino)sebacic acid. αα′-Dibromosebacic acid was treated with 33% (w/v) ethanolic dimethylamine (5 moles) at 37° in a sealed tube for 3 days. The contents were evaporated and the residue recrystallized from aqueous ethanol. The *product* melted at 268–269° with decomposition, varying slightly with rate of heating (yield 85%). (Found: C, 57·7; H, 9·6; N, 9·5. $C_{14}H_{28}O_4N_2$ requires C, 58·3; H, 9·7; N, 9·7%.)

1:10-*Diaminodecane*-1:10-*dicarboxylic acid.* Decane-1:10-dicarboxylic acid was prepared by a method based on that described by Ruhoff (1936) for the preparation of tridecanoic acid. Decamethylene dibromide (1 mole), KCN (3 moles) and 95% (v/v) ethanol were refluxed for 20 hr. on the water bath. KCN (3 moles) was added at the end of this time and the mixture was refluxed for a further 9 hr. After adding 85% (w/v) aqueous KOH (4 moles) and refluxing for 24 hr. the mixture was steam-distilled, acidified, steam-distilled again, and the precipitated decane-1:10-dicarboxylic acid was filtered off (yield 75%). M.p. 118–123°. It was converted into 1:10-*dibromodecane*-1:10-*dicarboxylic acid* with $SOCl_2$ (2·4 moles) and Br_2 (2·1 moles) (Guha & Sankaran, 1946). The product (57%) was recrystallized from benzene–light petroleum (b.p. 60–80°) and melted at 115–118°. Mixed with decane-1:10-dicarboxylic acid (m.p. 118°) it melted at 85–100°. (Found: C, 37·0; H, 5·0; Br, 41·2. $C_{12}H_{20}O_4Br_2$ requires C, 37·1; H, 5·2; Br, 41·3%.) This bromo acid was aminated with powdered $(NH_4)_2CO_3$ and aqueous NH_3 (sp.gr. 0·880). The *product* melted at 295–297° (yield 70%). (Found: C, 54·7; H, 9·3; N, 10·7. $C_{12}H_{24}O_4N_2$ requires C, 55·3; H, 9·2; N, 10·8%.)

1:10-*Bis(dimethylamino)decane*-1:10-*dicarboxylic acid* was prepared by treating the same bromo acid with 33% (w/v) ethanolic dimethylamine (12 moles) in a sealed tube at 37° for 5 days. M.p. of *product* was 248–250° with decomposition (yield 49%). (Found: C, 60·4; H, 10·0; N, 8·6. $C_{16}H_{32}O_4N_2$ requires C, 60·7; H, 10·1; N, 8·9%.)

1:12-*Diaminododecane*-1:12-*dicarboxylic acid.* The *tetraethyl ester of* 1:12-*diacetamidododecane*-1:1:12:12-*tetracarboxylic acid* was prepared in 33% yield from acetamidomalonic ester (2 moles) by refluxing with decamethylene dibromide (1·3 moles) and sodium ethoxide (2 moles) in anhydrous ethanol for 6 hr. The *product* had m.p. 93–94°. Mixed with acetamidomalonic ester (m.p. 95–97°) it softened at 81° and melted at 87–93°. (Found: C, 58·7; H, 8·6; N, 5·0. $C_{28}H_{48}O_{10}N_2$ requires C, 58·7; H, 8·9; N, 4·9%.) This material was hydrolysed with 48% (w/v) HBr for 6 hr. The resulting free amino acid (66% yield) was precipitated from an aqueous solution of its hydrobromide by the addition of excess 2N-NH_3. Since the free amino acid could not be recrystallized because of its insolubility, it was purified through its *hydrochloride*. This melted at 291–293° with decomposition. (Found: C, 45·9; H, 8·3; N, 8·0. $C_{14}H_{28}O_4N_2$, 2HCl requires C, 46·5; H, 8·3; N, 7·8%.)

NN′-*Diglycyl*-αα′-*diaminosuberic acid.* This was prepared by bromoacetylation of αα′-diaminosuberic acid, followed by amination of the product. Although the tripeptide gave

a single spot on paper chromatography and yielded only glycine and αα′-diaminosuberic acid on hydrolysis, insufficient was available to give satisfactory analytical figures. On heating, it started to char at 205° and melted with decomposition at 335–360°.

NN′-Diglycyl-αα′-diaminosebacic acid. This was prepared by coupling *N-p*-toluenesulphonylglycyl chloride (2·05 moles) with diethyl αα′-diaminosebacate (2 moles) in anhydrous ether. The ester was obtained by treatment of diaminosebacic acid with ethanol and HCl. Diethyl *NN′-p*-toluenesulphonylglycyl-αα′-diaminosebacate, was recrystallized from ethyl acetate–light petroleum (b.p. 60–80°) to constant m.p. 146–147°, and hydrolysed with N-NaOH (2 moles), to yield NN′-p-*toluenesulphonylglycyl-αα′-diaminosebacic acid*, which was recrystallized from methanol (m.p. 161–162°, yield 20%). (Found: N, 8·5. $C_{28}H_{38}O_{10}N_4S_2$ requires N, 8·6%.)

The *N-p*-toluenesulphonyl tripeptide was treated with Na (12 atoms) in liquid NH_3 to yield *NN′*-diglycyl-αα′-diaminosebacic acid which was precipitated by the addition of 25% mercuric acetate and 10% Na_2CO_3 according to the method of Neuberg & Kerb (1912), to free it from contaminating ammonium salts. The basic Hg complex was decomposed with H_2S. The *tripeptide* (yield 50%) charred at 250° but, did not melt below 360°. (Found: N, 16·2. $C_{14}H_{26}O_6N_4$ requires N, 16·2%.)

Chromatography of compounds

The identity and purity of all the amino acids and peptides prepared above were checked by chromatography. The R_F values are given in Table 1.

BACTERIAL TESTS AND DISCUSSION

The compounds synthesized above were tested as growth inhibitors against *Corynebacterium diphtheriae*, *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*), *Escherichia coli*, and *Streptococcus pyogenes* growing in Hedley Wright (1933) broth. The results are given in Table 2.

Since αα′-diaminosuberic acid inhibited growth of *C. diphtheriae* at a concentration of 25 mg./100 ml., and also showed some activity against the other three organisms, it was further tested against *C. diphtheriae* in the synthetic medium of Evans, Happold & Handley (1939), but under these conditions it failed to inhibit growth. It also failed to protect mice against *Strep. pyogenes* when injected intraperitoneally in doses approaching the toxic level. αα′-Diaminosebacic acid, tested in the same way, also failed to show chemotherapeutic activity.

It is of interest that the only homologues showing significant antibacterial activity were those with 8 and 10 carbon atoms. It was at first thought that the low activity of the higher homologues of diaminopimelic acid might be related to their insolubility, but 1:10-bis(dimethylamino)decane-1:10-dicarboxylic acid, αα′-diaminosebacic acid diamide, αα′-

Table 1. *Chromatographic properties of αα′-diaminopimelic acid and its homologues*

The phenol and collidine solvents are the mixtures described by Dent (1948). Whatman no. 4 paper was used with descending flow and substances were located with ninhydrin.

Substance	R_F Phenol–water–NH_3	R_F Collidine
αα′-Diaminoadipic acid	0·22	0·03
αα′-Diaminopimelic acid	0·33	0·03
αα′-Diaminosuberic acid	0·44	0·03
αα′-Diaminoazelaic acid	0·55	0·05
αα′-Diaminosebacic acid	0·65	0·07
1:10-Diaminodecane-1:10-dicarboxylic acid	0·76	0·13
1:12-Diaminododecane-1:12-dicarboxylic acid	0·81	0·21
NN′-Diglycyl-αα′-diaminosuberic acid	0·67	0·03
NN′-Diglycyl-αα′-diaminosebacic acid	0·78	0·05

Table 2. *Concentrations of synthetic amino acids required to prevent visible growth in Hedley Wright broth overnight at* 37°

Compound	mg./100 ml. *Strep. pyogenes*	*Staph. aureus*	*Esch. coli*	*C. diphth.*
αα′-Diaminoadipic acid	> 50	> 50	> 50	—
αα′-Diaminopimelic acid	>500	>500	>500	—
αα′-Diaminosuberic acid	50	> 50	> 50	25
αα′-Diaminoazelaic acid	>500	>500	>500	500
αα′-Diaminosebacic acid	Inactive in sat. solution			~ 10
1:10-Diaminodecane-1:10-dicarboxylic acid	Inactive in sat. solution			
1:12-Diaminododecane-1:12-dicarboxylic acid	Inactive in sat. solution			
αα′-Bis(dimethylamino)sebacic acid	>500	>500	>500	>500
1:10-Bis(dimethylamino)decane-1:10-dicarboxylic acid	>500	>500	>500	300
αα′-Diaminosebacamide	500	>500	>500	500
NN′-Diglycyl-αα′-diaminosebacic acid	>200	>200	>200	—

bis(dimethylamino)sebacic acid, *NN'*-diglycyldiaminosuberic acid and *NN'*-diglycyldiaminosebacic acid were all readily soluble in water, but were less active than diaminosuberic acid. It is also possible that permeability considerations play an important part in the assimilation of these compounds.

SUMMARY

1. Methods are described for the synthesis of six αα'-diaminodicarboxylic acids, homologues of αα'-diaminopimelic acid.

2. These amino acids and some of their derivatives have been tested as antibacterial agents.

The author wishes to thank Miss J. Horton for performing the biological testing. The author is also indebted to the Commonwealth of Australia Scientific and Industrial Research Organization for a scholarship held during the course of this work. Dr T. S. Work is thanked for advice throughout the course of this work and the Medical Research Council for hospitality during the tenure of the CSIRO scholarship.

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Diaminopimelic Acid Decarboxylase in Cells and extracts of *Escherichia coli* and *Aerobacter aerogenes*

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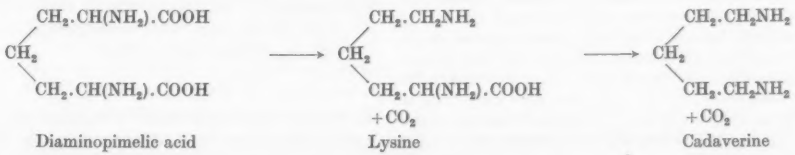
(*Received* 14 *May* 1954)

αε-Diaminopimelic acid is a constituent of many bacteria and blue-green algae, but is absent from all other organisms so far examined. It has been found in certain cellular proteins, in insoluble cell-wall fractions, in the free state and in an extracellular peptide (Work, 1951; Work & Dewey, 1953; Gendre & Lederer, 1952; Holdsworth, 1952; Salton, 1953; Blass, Lecomte & Macheboeuf, 1951; Work, 1949; Pauletta & Defranceschi, 1952; Powell & Strange, 1953). In order to gain some knowledge of the metabolism of diaminopimelic acid, a search was made for a bacterial enzyme which would attack it. The possibility of decarboxylation was first considered, and *Escherichia coli* was the organism originally chosen since it was known to contain a large number of amino acid decarboxylases (Gale, 1946). Using the acidic conditions of growth and enzyme assay found by Gale to produce optimum decarboxylase activity, no decarboxylation of diaminopimelic acid was obtained. Attention was then directed to the transamination reaction which occurs in an alkaline medium (Feldman & Gunsalus, 1950). Under alkaline conditions, freeze-dried cell suspensions of *Esch. coli* converted diaminopimelic acid into lysine and cadaverine, and it was apparent that decarboxylation, and not transamination, had taken place.



The difference in pH optima for these two successive decarboxylations, and the differential distribution of the two activities in various strains of *Esch. coli* suggested the existence of two enzymes, one decarboxylating diaminopimelic acid and the other decarboxylating lysine (Dewey & Work, 1952*a*).

This paper describes the enzyme diaminopimelic acid decarboxylase, which converts diaminopimelic acid into lysine and carbon dioxide. The enzyme was first investigated in a strain of *Esch. coli*, but later a strain of *Aerobacter aerogenes* proved to be a richer and more convenient source of the enzyme. The optimum conditions for assay and production of the enzyme in both organisms are described, together with the preparation and properties of cell-free extracts from *Aero. aerogenes*. The work has already been described in brief (Dewey & Work, 1952*b*; Dewey, Hoare & Work, 1953).

METHODS

Organisms

Escherichia coli. This was a strain from the collection of this Medical School. It was a faecal type, giving the following reactions: methyl red, positive; Voges-Proskauer (reaction for acetoin formation), negative; indole, positive; no growth in citrate.

Aerobacter aerogenes ('strain 1' from the collection of the Medical Research Council Unit for Chemical Microbiology, Cambridge) gave the following reactions: methyl red, negative; Voges-Proskauer, positive; indole, negative; growth in citrate. (These differential coliform tests were carried out as recommended in Report no. 71, 'The Bacteriological Examination of Water Supplies', London: H.M. Stationery Office, 1939).

Growth media

Except where otherwise stated, the following media were used:

Broth contained: proteose peptone (Difco Laboratories Inc., Detroit 1, Michigan), 5 g.; yeast extract (Marmite Food Extract Co. Ltd., London, E.C. 3), 2 g.; Oxoid 'Lab-Lemco' (Oxo Ltd., London, E.C. 4), 1 g.; NaCl, 5 g.; glucose (autoclaved separately), 5 g.; water to 1 l.

Minimal medium 1. This contained: K_2HPO_4, 7 g.; KH_2PO_4, 3 g.; trisodium citrate, 0·5 g.; $(NH_4)_2SO_4$, 1·0 g.; $MgSO_4$, $7H_2O$, 0·01 g.; $FeSO_4$, $7H_2O$, trace; glucose (autoclaved separately), 2·0 g.; water to 1 l. (final pH 7·4).

Minimal medium 2. This was similar except that the glucose concentration was 20 g./l. and $MgSO_4$, $7H_2O$, 0·05 g./l.

Growth and harvesting of organisms

The organisms were grown either in 160 ml. of medium in Roux bottles, or in 2–8 l. batches in suitable glass bottles aerated either by a slow stream of air or by rapid rotation on inclined rollers. Inocula were 24 hr. cultures grown on an amount of medium representing 1–2·5% of the final volume used. Unless otherwise stated, *Esch. coli* was grown at 37° and harvested after 12·5 hr.; *Aero. aerogenes* was grown at 25° and harvested after 24 hr. The organisms were harvested by centrifuging, washed with water and acetone-dried by the method of Gale & Epps (1944). The dried cells were stored at −10°.

Assays of enzyme activity

Buffers. All buffers were made with glass-distilled water and unless otherwise stated were 0·1 M mixtures of K_2HPO_4 and KH_2PO_4. Assays on *Esch. coli* and *Aero. aerogenes* preparations were carried out at pH 7·2 and 6·8 respectively.

Substrates. Natural diaminopimelic acid was prepared from toxin-extracted *Corynebacterium diphtheriae* (Work, 1951), and also from the culture fluid of a lysine auxotroph of *Esch. coli* (Work & Denman, 1953); solutions (0·025 M) in buffers of appropriate pH were used. L-Lysine monohydrochloride (0·033 M) was also dissolved in buffer.

Manometric technique. Decarboxylation was followed manometrically in the Warburg apparatus at 37°; the gas phase was air unless otherwise stated. The main compartment contained either 40 mg. of dried cell preparation suspended in 2 ml. buffer or a cell-free enzyme preparation extracted from 40 mg. of dried cells dissolved in 2 ml. of buffer; 0·1 ml. of an aqueous solution of barium pyridoxal phosphate (0·1 mg./ml.) was also added. Substrate solution (0·5 ml.) was tipped from a side arm after equilibration. The reaction rate was measured from the slope of the linear portion of the plot of gas evolution against time. At pH 6·8 and 7·2 significant amounts of CO_2 were retained in the aqueous phase and were not measured in rate determinations. The enzyme activity was expressed as $Q_{CO_2} = \mu l.$ CO_2 evolved/hr./mg. of dried cells at 37° (not corrected for CO_2 retention). To obtain a quantitative estimation of CO_2 evolution, 0·1 or 0·2 ml. of 8 N-H_2SO_4 was placed in the second side arm and was tipped into the main compartment when the gas evolution had ceased.

Chemical estimations

Total N was measured by the micro-Kjeldahl method. The optical density (E) at 280 mμ., measured in a Beckman spectrophotometer, was used for rough estimations of protein in solution.

Paper chromatography

The enzymic reaction products were examined after deproteinization. When acid had been tipped, the precipitated protein was removed by centrifuging and the solution neutralized with $Ba(OH)_2$; in other cases, 1 vol. of ethanol was added, and the mixture was boiled and centrifuged. Supernatant solutions (equiv. 40–50 μl. of reaction mixture) were examined by paper chromatography for ninhydrin-reacting components. Phenol (NH_3 atmosphere) was used as one solvent, and collidine–lutidine (Dent, 1948) or butanol–acetic acid (Campbell, T. S. Work & Mellanby, 1951) was the second solvent used when two-dimensional chromatograms were required.

RESULTS

Properties of diaminopimelic acid decarboxylase in whole-cell suspensions of *Esch. coli* and *Aero. aerogenes*

Products of decarboxylation

Both the organisms under investigation contained lysine decarboxylase when grown on the lysine-

containing nutrient broth. The decarboxylation of diaminopimelic acid was the rate-controlling step; the lysine formed did not accumulate but was rapidly converted into cadaverine by the lysine decarboxylase, which was considerably more active than diaminopimelic acid decarboxylase, even under the neutral conditions of assay. The rate of carbon dioxide evolution was thus proportional to the diaminopimelic acid decarboxylase activity, and 2 moles of carbon dioxide were evolved/mole of diaminopimelic acid decarboxylated. Using a suspension of dried *Esch. coli* grown on broth, the observed gas evolution (498 μl.) was 89 % of the theoretical amount (560 μl.) expected from the removal of 2 moles of carbon dioxide from 0·5 ml. of 0·025 M diaminopimelic acid. The gas evolved was shown to be carbon dioxide since no volume change occurred when the reaction was carried out in flasks containing 20 % (w/v) potassium hydroxide in the centre wells. The other reaction product, cadaverine, was identified by paper chromatography. Added L-lysine was also quantitatively converted into cadaverine, proving the activity of lysine decarboxylase at pH 6·8; no reaction occurred with D-lysine. *Esch. coli*, when grown on minimal medium 1, had negligible amounts of lysine decarboxylase (Dewey & Work, 1952*a*), and the gas evolution corresponded to the loss of 1 mole of carbon dioxide/mole of diaminopimelic acid; paper chromatography showed that lysine was the only ninhydrin-reacting end product.

Establishment of optimum conditions for enzyme assay

Using *Esch. coli* grown on broth, acetone drying of the cells was essential in order to obtain maximum diaminopimelic acid decarboxylase activity. These dried cells showed no respiration, and as the rate of carbon dioxide evolution was not changed by substituting air for nitrogen, all assays were carried out with air as the gas phase.

The optimum pH for the decarboxylation of diaminopimelic acid by dried *Aero. aerogenes* was found to be 6·8 and this pH was used in all tests on this organism. Enzyme activity was halved by increasing the buffer strength to M, but was not affected by reducing the concentration below 0·1 M.

The rate of decarboxylation of diaminopimelic acid by dried *Esch. coli* grown on broth was doubled by adding either pyridoxal phosphate or a boiled suspension of *Esch. coli*. Different batches of *Aero. aerogenes* grown on minimal medium 1 showed different degrees of activation by pyridoxal phosphate; in some batches there was no measurable effect, in others up to 80 % increase in activity was obtained.

Effect of variation of growth conditions on enzyme activity

The diaminopimelic acid decarboxylase activities of *Esch. coli* and *Aero. aerogenes* were affected differently by the phase of growth at which they were harvested. With *Esch. coli* (Fig. 1) enzyme activity was greatly influenced by growth time, although the maximal activity attainable on a given medium was almost independent of growth temperature. With *Aero. aerogenes* grown at 25° on either type of minimal medium, growth time had little effect on diaminopimelic acid decarboxylase activity (Table 1), but lysine decarboxylase activity increased with duration of growth. Similar results were obtained with cells grown on broth.

Changes in composition of the growth medium were made with the following aims: (*a*) to raise the concentration of diaminopimelic acid decarboxylase; (*b*) to reduce the concentration of lysine decarboxylase; (*c*) to increase the total yield of cells. Replacement of broth by minimal medium had no effect on diaminopimelic acid decarboxylase

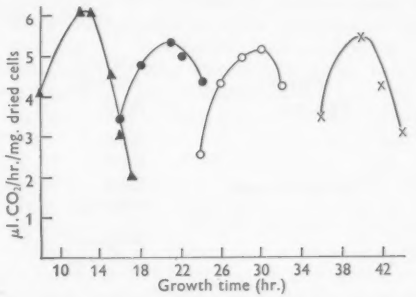


Fig. 1. Effect of growth time and growth temperature on diaminopimelic acid decarboxylase activity in *Esch. coli*: ▲, cells grown at 37° on nutrient broth and tested at pH 7·2; cells grown at 18° (×), at 25° (○) and at 30° (●), on a salt–glucose medium (Stephenson, 1949), tested at pH 6·8.

Table 1. *Effect of growth time on decarboxylase activities of* Aero. aerogenes

For conditions, see text.

Medium	Growth time (hr.)	Yield of dried cells (g./l.)	Q_{CO_2} at pH 6·8: Diaminopimelic acid	Q_{CO_2} at pH 6·8: Lysine
Minimal 1	22	0·42	9·4	—
	24	0·45	9·8	—
	26	0·44	9·4	—
	28	0·42	9·9	—
Minimal 2	15	0·98	6·8	1·5
	24*	1·37	7·8	3·2
	28	1·48	6·4	4·2

* Near end of logarithmic growth.

(Table 2), but virtually eliminated lysine decarboxylase activity in *Esch. coli* (assayed at pH 7·2), and considerably reduced it in *Aero. aerogenes* (assayed at pH 6·8). Diaminopimelic acid decarboxylase activity of both organisms was not changed by the addition of pyridoxin or diaminopimelic acid to the growth medium (Table 2) and in *Aero. aerogenes* it was unaffected by changes in pH between 6·0 and 7·6 (Table 3). *Esch. coli* grown on broth showed a sixfold drop in rate of decarboxylation of diaminopimelic acid with rise in pH from 5·0 to 8·0. As lysine decarboxylase activity was shown by Gale & Epps (1944) to decrease markedly over the same pH range, the apparent decrease in activity of *Esch. coli* may be partly attributed to lysine decarboxylase becoming the rate-limiting factor in the tests made on cells grown at higher pH.

The most effective method of raising the yield of cells without increasing lysine decarboxylase was to increase the glucose concentration of the minimal medium. This had no effect on diaminopimelic acid decarboxylase, although the figures in Table 3 showed an apparent fall. In effect, this was an unexplained fall in activity which occurred in the cells of the stock culture at the time that the change from minimal medium 1 to 2 was made.

Preparation and fractionation of cell-free extracts containing diaminopimelic acid decarboxylase

Extraction of enzyme

No diaminopimelic acid decarboxylase activity was extracted from dried *Esch. coli* after incubation for 2 hr. at 37° with buffers between pH 4 and 8. Disintegration of dried cells in 0·1M phosphate buffer (pH 6·8) produced active extracts which maintained their activity in solution after centrifuging for 40 min. at 25 000 *g*. The methods of disintegration were: shaking (10 min.) with ballo-

Table 2. *Effect of change in composition of growth medium on diaminopimelic acid decarboxylase activity*

For conditions of tests, see text.

Organism	Control medium	Test medium (wt. of nutrient added/100 ml. medium)	Q_{CO_2} control cells	Q_{CO_2} test cells
Esch. coli	Broth	Broth + diaminopimelic acid (100 mg.)	4·0*	4·0*
	Broth	Broth + pyridoxin (10 mg.)	4·8*	3·9*
	Broth	Minimal 1	3·7*	3·0
Aero. aerogenes	Broth	Minimal 1	22*	9·0
	Minimal 1	Minimal 1; ($MgSO_4$, $7H_2O$ increased to 10 mg.)	9·0	8·0
	Minimal 2	Minimal + pyridoxin (10 mg.)	5·3	6·2
	Minimal 2	Diaminopimelic acid added† (10 mg.)	5·2	5·3

* These figures are twice the Q_{CO_2} for diaminopimelic acid decarboxylase because of the presence of lysine decarboxylase in broth cultures.

† Concentrated mutant medium (Work & Denman, 1953).

Table 3. *Effect of* pH *and aeration of growth medium on diaminopimelic acid decarboxylase activity*

For conditions, see text.

Organism	Growth conditions	Initial pH	Final pH	Yield of dry cells (g./l.)	Q_{CO_2}
Esch. coli	Buffered broth. Normal aeration	8	8	0·40	1·0
		7	7	0·47	2·1
		6	6	0·40	4·5
		5	5	0·29	6·2
Aero. aerogenes	Minimal medium 1. Normal aeration	7·4	6·7	0·20	9·4
		6·9	6·8	0·24	9·5
		6·6	6·6	0·13	10·2
		6·0	6·4	0·11	9·7
Aero. aerogenes	Minimal medium 2. Normal aeration	7·6	6·0	1·14	6·1
		7·4	5·7	1·25	5·7
		6·9	5·0	0·92	6·7
		6·1	5·0	1·01	6·5
Aero. aerogenes	Minimal medium 2. Poor aeration*	7·4	6·0	0·80	5·4
Aero. aerogenes	Minimal medium 2. Vigorous aeration	7·4	5·2	1·26	5·2

* 250 ml. batches.

tini beads in a Mickle cell-disintegrator (Mickle, 1948); ultrasonic disintegration (Smith & Stumpf, 1946); grinding with an abrasive (Dockstader & Halvorson, 1950). Many of the cell-free extracts were prepared by the last method, using to 1 part of cells, 2 parts of Filter-cel (Johns Manville Co., Ltd., London), and 6 parts of buffer. The mixture was ground by hand for 15 min. in a pestle and mortar. On centrifuging, this gave a supernatant containing 86% of the original diaminopimelic acid decarboxylase activity.

Fractionation of enzyme

Since most of the lysine decarboxylase was extracted with the diaminopimelic acid decarboxylase, it was necessary to find a method of separating the two enzymes before further studies could be made. Separation was studied on cell-free extracts of *Aero. aerogenes* grown on broth so that measurable lysine decarboxylase would be present. Fractionation with inorganic salts was not possible because they readily inactivated diaminopimelic acid decarboxylase (Table 5). Fractional precipitation with acetone at $-10°$ produced no separation. At 0°, the bulk of the lysine decarboxylase was precipitated between 45 and 50% (v/v) acetone, while only 11% of the original diaminopimelic acid decarboxylase was found in this fraction (Table 4).

Fractional extraction with acetone. By making use of the lower solubility of lysine decarboxylase in acetone–buffer mixtures it was possible to extract preferentially diaminopimelic acid decarboxylase from disintegrated cells. By grinding dried *Aero. aerogenes* with abrasive and 0·1 M phosphate buffer (pH 6·8) containing 55% (v/v) acetone, centrifuging the extract and adding acetone to 80% (v/v)

Table 4. *Relative solubilities of diaminopimelic acid and lysine decarboxylases in mixtures of acetone with* 0·1 M *potassium phosphate buffer* (pH 6·8)

Starting solution; 20 ml. of cell-free buffer extract from 500 mg. of acetone-dried *Aero. aerogenes*; acetone at 0° added slowly with stirring. Solution centrifuged, supernatant solution further fractionated. Solids freed from acetone at 0° and tested for enzyme activity.

Acetone concn. range (%, v/v)		Percentage recovery: Diaminopimelic acid decarboxylase	Lysine decarboxylase	Protein ($E_{280\ m\mu}$)
0–35		0	1	3
35–40		0	1·5	2
40–45	*	0	4	3
45–50		11	40	24
50–55		24	3	25
55–60	*	19·5	2·5	11
60–65		5·5	1	8·9
65–70		3·5	0	4

* Brackets indicate separate experiments. Mean figures given in overlap ranges.

to the supernatant, a precipitate was obtained which, after removal of acetone, contained 40% of the original diaminopimelic acid decarboxylase but only 1% of the lysine decarboxylase of the cells. After reducing the lysine decarboxylase content by growing the cells on minimal medium 1, a diaminopimelic acid decarboxylase preparation was obtained containing no demonstrable lysine decarboxylase. However, recovery of diaminopimelic acid decarboxylase from these cells was only 20%; this figure was restored to 40% by reducing the acetone content of the extraction mixture to 48% (v/v). Variation of the pH between 5·7 and 7·2 had no effect on the enzymic activity of the preparation, although the total protein extracted rose with increasing pH. Outside these limits, the activity fell sharply. Therefore, pH 5·8 was chosen for subsequent extractions.

The standard partially purified cell-free preparation of diaminopimelic acid decarboxylase was made in the following manner from *Aero. aerogenes* grown on minimal medium. In a mortar of uniform curvature dried cells (1 g.) were mixed to a thick paste with 5–6 ml. of extraction mixture (acetone, 48 ml.; 0·025 M phosphate buffer (pH 5·8), 52 ml.). Filter-cel (1 g.) was added in successive small portions and grinding was continued for 15 min. The contents of the mortar were washed into centrifuge tubes with extraction mixture (overall volume used, 75 ml.), the tubes were covered and centrifuged for 20 min., at 3000 rev./min. using an International Centrifuge size 1 (Gallencamp and Co., London). All these operations were carried out at $+2°$. The supernatant liquid (66 ml.) was cooled to $-10°$ and 100 ml. of acetone at $-10°$ were added. After standing for 10 min. the precipitate was collected by centrifuging and freed from acetone *in vacuo* at $+2°$. This precipitate was dissolved in 10 ml. of 0·1 M buffer (pH 6·8). This solution (1 ml., containing 0·344 mg. N) produced carbon dioxide from diaminopimelic acid at 473 μl./hr. Since the original cells contained 6·5% N and had Q_{CO_2} 11·0, the purification of enzyme achieved was 8·1 (based on N content). Total enzyme recovery was 43%.

Properties of partially purified diaminopimelic acid decarboxylase from Aero. aerogenes

The properties of the enzyme were investigated in the standard preparation made as described in the previous section. The amount of enzyme used in each test was 1 ml.

Enzyme characteristics. The effect of change in pH on observed enzyme activity is shown in Fig. 2. The curve has a sharp maximum at pH 6·8, which is similar to that shown by dried-cell suspensions. Since no corrections have been made for increasing retention of carbon dioxide with rise in pH, the curve does not represent the true effect of pH on

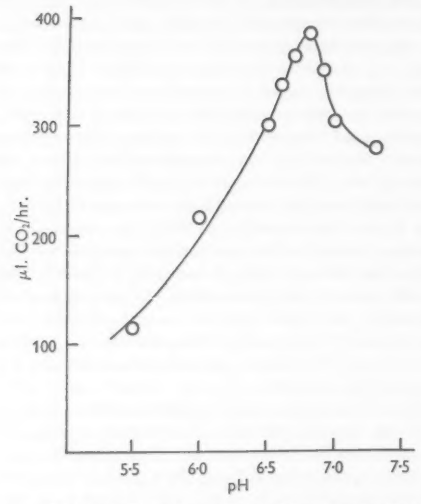


Fig. 2. Effect of pH on the activity of diaminopimelic acid decarboxylase in purified extracts of *Aero. aerogenes*.

enzyme activity, but only the observed effect which is a combination of changes in enzyme activity and carbon dioxide retention. Because of the sharpness of the peak, the true optimum would not be far from the observed value.

The retention of carbon dioxide has prevented any calculation of Michaelis constant, but judging from the observed velocities, the substrate concentration giving half-maximum velocity is about 0·0028 M.

A straight-line relationship was found between enzyme concentration and rate of carbon dioxide evolution. The plot of the two functions did not pass through the origin, but cut the ordinate at a point representing 30 μl. CO_2/hr. This may represent the carbon dioxide held by the buffer before saturation is reached.

Addition of pyridoxal phosphate caused variable increases in activity of different preparations, the magnitude of the effect being similar to that observed with the suspension of the particular batch of cells from which the enzyme preparation was made. Addition of boiled cell suspensions of dried *Aero. aerogenes* had no effect on enzyme activity in the presence of added pyridoxal phosphate. The chelating agent 'Versene' (ethylenediaminetetraacetic acid) was without effect on enzyme activity, suggesting that no metal cofactor is required.

Partial irreversible inactivation occurred on dialysis. This could be prevented by the addition of 2:3-dimercaptopropanol ($M \times 10^{-3}$) to the dialysis liquid. No dissociation of pyridoxal phosphate resulted from dialysis.

Table 5 shows the effect of various salts and enzyme inhibitors on decarboxylase activity. The most potent inhibitors were cupric sulphate and *p*-chloromercuribenzoate. Because of the susceptibility to copper, the enzyme was at times inhibited by water from the laboratory copper still, necessitating the use of glass-distilled water throughout. The inhibition caused by *p*-chloromercuribenzoate was reversed to the extent of 70% by adding glutathione 5 min. after mixing enzyme and inhibitor (Table 6). No inhibition was obtained with glycine, DL-alanine, DL-valine, DL-isoleucine, or DL-aspartic acid when present in concentrations four times that of the substrate. High concentrations of DL-glutamic acid inhibited the enzyme; the degree of inhibition was dependent on the molar ratio of glutamic acid to diaminopimelic acid (a ratio of 10/1 gave 50% inhibition). For tests on inhibition by homologues of diaminopimelic acid see Table 7.

Specificity. Diaminopimelic acid decarboxylase is highly specific for the naturally occurring form of substrate (Table 7). The mean value (263 μl.) for eleven estimations of carbon dioxide evolution from 0·005 m-moles of natural diaminopimelic acid was 94% of the theoretical. Various unfractionated samples of synthetic diaminopimelic acid gave

Table 5. *Action of inhibitors on partially purified diaminopimelic acid decarboxylase*

For conditions of tests, see text.

Inhibitor	Molar concentration giving 50% inhibition
Ammonium sulphate	$2{\cdot}5 \times 10^{-1}$
Magnesium sulphate	$1{\cdot}0 \times 10^{-1}$
Copper sulphate	$3{\cdot}0 \times 10^{-5}$
Potassium cyanide*	$4{\cdot}2 \times 10^{-4}$
Semicarbazide	$1{\cdot}2 \times 10^{-4}$
Iodoacetamide	$2{\cdot}0 \times 10^{-3}$
Iodosobenzoate	$1{\cdot}4 \times 10^{-4}$
p-Chloromercuribenzoate†	$5{\cdot}0 \times 10^{-5}$
p-Arsinosobenzamide	$1{\cdot}0 \times 10^{-3}$

* Reversed by dialysis.
† Reversed by glutathione (see Table 6).

Table 6. *Reversal by glutathione of* p*-chloromercuribenzoate inhibition of diaminopimelic acid decarboxylase*

0·3 ml. of 0·1 M glutathione added to one of two duplicate Warburg flasks 5 min. after adding the inhibitor. The resulting enzyme activity is expressed as % of control.

p-Chloromercuribenzoate concentration (M)	% Enzyme activity present	
	No glutathione	Glutathione added
Nil	100	95
$3{\cdot}75 \times 10^{-5}$	68	72
$1{\cdot}25 \times 10^{-4}$	4	74
$3{\cdot}75 \times 10^{-4}$	0	70

carbon dioxide output 45–48% of theoretical and the reaction mixture contained lysine and unchanged diaminopimelic acid. The enzyme had no effect on any of the common naturally occurring amino acids. Several members of the homologous series of diaminodicarboxylic acids and related substances were also tested with negative results.

Use of diaminopimelic acid decarboxylase for the identification of diaminopimelic acid in natural materials

Diaminopimelic acid could not be estimated enzymically in acid hydrolysates of micro-organisms owing to enzyme inhibition by constituents of the acid hydrolysate, even after complete removal of mineral acid by ion-exchange resins or trioctylamine (Hughes & Williamson, 1951). After fractionation in an electrodialysis cell (Work & Dewey, 1953), diaminopimelic acid in the neutral fraction was completely decarboxylated as shown by examination of the reaction products by paper chromatography. Under the conditions used, there was a variable loss of diaminopimelic acid to the cathode compartment, therefore the true content of diaminopimelic acid could not be estimated. Some figures obtained with a few organisms are given in Table 8; they are not corrected for cathode losses and are thus only minimal values.

The figures on the *Streptomyces* sp. are included since the organism was shown by paper chromatography to contain an unidentified amino acid differing only slightly from diaminopimelic acid in

Table 7. *Specificity of diaminopimelic acid decarboxylase for structural analogues of diaminopimelic acid and the effect of analogues as inhibitors of the enzyme*

Inhibition experiments were conducted with analogue and diaminopimelic acid at equimolar concentrations.

Compounds of the type $HOOC.CH(NH_2).(CH_2)_x.CH(NH_2).COOH$	Source	Decarboxylation (%)	Inhibition (%)
$x=0$. Diaminosuccinic acid	Synthetic	0	0
$x=1$. Diaminoglutaric acid	Synthetic	0	0
$x=2$. Diaminoadipic acid	Synthetic[1]	0	0
$x=3$. Diaminopimelic acid	Synthetic[1]	45–48 (mean 46)	—
	C. diphtheriae[2]	90–98 (mean 94)	—
	Esch. coli (mutant medium)[3]	85–95 (mean 90)	—
$x=4$. Diaminosuberic acid	Synthetic[1]	0	0
$x=5$. Diaminoazelaic acid	Synthetic[1]	0	0
$x=6$. Diaminosebacic acid	Synthetic[1]	0	0
$x=8$. 1:10-Diaminodecan-1:10-dicarboxylic acid	Synthetic[1]	0	0
$x=10$. 1:12-Diaminododecan-1:12-dicarboxylic acid	Synthetic[1]	0	0
Other compounds			
ε-*C*-Methyllysine	Synthetic[4]	0	0
αε-Diamino-β-hydroxypimelic acid	*Pseudomonas tabaci* toxin[5]	0	15
Lanthionine	Wool[6]	0	0
meso-Lanthionine	Subtilin[7]	0	0
Pyridine-2:6-dicarboxylic acid (dipicolinic acid)	*B. megatherium* germinating spores[8]	0	45
'Cysteine sulphinic acid'	L-Cystine[9]	0	0

[1] Simmonds, 1954.
[2] Work, 1951.
[3] Work & Denman, 1953.
[4] McLaren & Knight, 1951.
[5] Woolley, Schaffner & Braun, 1952.
[6] Horn, Jones & Ringel, 1941.
[7] Alderton & Fevold, 1951.
[8] Powell, 1953.
[9] Lavine, 1936.

Table 8. *Diaminopimelic acid content of the neutral fraction obtained in electrodialysis cell from hydrolysates of micro-organisms, estimated by diaminopimelic acid decarboxylase*

Organism	Amount tested (equiv. dry wt. of cells in mg.)	Diaminopimelic acid: mg. found assuming 94% decarboxylation	Diaminopimelic acid: g./100 g. dry wt. of organism
Corynebacterium diphtheriae	80	0·40	0·50
C. diphtheriae	100	0·53	0·53
C. diphtheriae	160	0·70	0·45
Brucella abortus	100	0·54	0·54
Shigella dysenteriae	100	0·19	0·19
Rhodopseudomonas palustris	180	0·55	0·31
Streptomyces sp.*	200	0	0
Streptomyces sp.* + 1·2 mg. diaminopimelic acid	200	1·2	0

* See text.

its chromatographic properties. This amino acid was not decarboxylated and was subsequently isolated and identified as a branched-chain higher homologue of diaminopimelic acid (Work, 1953).

DISCUSSION

The estimation of diaminopimelic acid decarboxylase activity in micro-organisms is complicated by two factors. First, the retention of carbon dioxide in the neutral buffer prevented a true measure of enzyme activity from the rate of gas evolution. Because of the variable proportions of carbon dioxide dissolved during the course of the reaction, no correction was made for this retention. The second difficulty in measuring the activity in whole cells is the presence of lysine decarboxylase. When lysine is rapidly decarboxylated, diaminopimelic acid decarboxylation becomes the rate-limiting step; but where the lysine decarboxylase was not very active, there was no method of assessing the meaning of the Q_{CO_2} in terms of rate of decarboxylation of diaminopimelic acid.

Preparation of purified cell-free extracts of diaminopimelic acid decarboxylase has been rendered difficult by the low enzyme activity in the bacteria examined. By comparison with lysine decarboxylase, where Gale (1946) reports $Q_{CO_2} > 400$, the values of 10 or less found for diaminopimelic acid decarboxylase are negligible. The change from *Esch. coli* to *Aero. aerogenes* as a source of enzyme had many advantages. The enzyme in the latter organism was not only more active; it also had a slightly lower pH optimum, which decreased carbon dioxide retention in buffer. In *Aero. aerogenes* the time of harvesting was without effect on enzyme activity, while with *Esch. coli* growth time was so critical that, in order to obtain reproducible results, a machine had to be constructed to give a uniform inoculum at a specified time (Dewey & Wallace, 1953).

The diaminopimelic acid decarboxylase content of *Aero. aerogenes* was independent of all changes made in the growth medium. It is thus likely that this enzyme is part of the constitutive equipment of the cell and is not an adaptive enzyme as are the six bacterial amino acid decarboxylases described by Gale (1946). The products of decarboxylation of these six amino acids are mostly bases, without known major metabolic function; they are probably by-products formed as an adaptive defence mechanism of the cell against acidic environments (Gale, 1946). On the other hand, the product of decarboxylation of diaminopimelic acid is the 'essential' amino acid lysine. Davis (1952) has shown that in *Esch. coli* (ATCC 9637) diaminopimelic acid is a precursor of lysine; here the enzyme responsible for the conversion is diaminopimelic acid decarboxylase, since it is missing from certain lysine auxotrophs of *Esch. coli* that accumulate diaminopimelic acid in their culture media (Dewey & Work, 1952*b*). The activity of diaminopimelic acid decarboxylase at neutrality and its presence in the cell at early phases of growth also distinguish it from the adaptive amino acid decarboxylases, which have acid pH optima and are formed towards the end of cell division. King (1953) has reported that valine and leucine are decarboxylated at pH 7 by *Proteus vulgaris* grown in the presence of either one of these amino acids. It is thus evident that both the neutral and the polar amino acids are susceptible to decarboxylation by bacteria, and that certain bacterial amino acid decarboxylases will operate at neutral reaction.

Although rigorous proof of the requirement for pyridoxal phosphate has not been obtained, the activation of the enzyme by pyridoxal phosphate and the reversible inhibition by cyanide suggest that this substance is the coenzyme. The studies on inhibition indicate that diaminopimelic acid decarboxylase is a very sensitive SH enzyme.

The high specificity of the enzyme for the natural substrate is similar to that of the other bacterial amino acid decarboxylases; the true optical specificity has not yet been investigated, as the three diasterio isomers of diaminopimelic acid are not yet available. Only half of the synthetic diaminopimelic acid was attacked, and the reaction mixture contained lysine and unchanged diaminopimelic acid; this indicates that the enzyme has a definite optical specificity. The lysine formed from decarboxylation of both natural and synthetic diaminopimelic acid was the L- form, since it was further decarboxylated to cadaverine by the bacterial lysine decarboxylase which was specific for L-lysine and unable to attack the D- form.

The use of diaminopimelic acid decarboxylase as an analytical tool for the estimation of diaminopimelic acid has been prevented by the easy inhibition of the enzyme, although, when a suitable means for quantitative removal of inhibitors is found, the enzymic method of assay should be applicable to biological materials. However, the method has already been of use in checking chromatographic evidence for the presence of diaminopimelic acid in micro-organisms (Work & Dewey, 1953) and, because of its specificity, it has helped in the identification of a naturally occurring homologue of diaminopimelic acid (Work, 1953).

SUMMARY

1. An enzyme decarboxylating diaminopimelic acid has been studied in single strains of *Escherichia coli* and of *Aerobacter aerogenes*.

2. The reaction products are L-lysine and carbon dioxide.

3. Changes in growth conditions indicated that the enzymic activity of the bacteria was unaffected by pH, composition or degree of aeration of the growth medium. In *Aero. aerogenes*, but not in *Esch. coli* it was also unaffected by phase of growth.

4. The pH optimum was 7·2 for *Esch. coli* and 6·8 for *Aero. aerogenes*.

5. Partially purified cell-free extracts of enzyme were obtained from *Aero. aerogenes* by fractional acetone extraction. The properties of the enzyme in such extracts were investigated.

6. Pyridoxal phosphate was required for maximum activity, and cyanide caused inhibition, which was reversed by dialysis.

7. The enzyme was strongly inhibited by copper and other SH reactive inhibitors. Glutathione reversed *p*-chloromercuribenzoate inhibition.

8. The enzyme was highly specific for natural diaminopimelic acid. Of those tested, no other naturally occurring amino acid or natural or synthetic homologue of diaminopimelic acid was decarboxylated. Synthetic diaminopimelic acid was decarboxylated to half the extent for the natural product.

We gratefully acknowledge gifts of cultures and chemicals from Dr E. P. Abraham, Dr G. Alderton, Dr B. D. Davis, Professor F. Dickens, Professor F. Egami, Professor C. Fromageot, Dr E. F. Gale, Dr H. Heath, Dr A. D. McLaren, Dr H. K. Mitchell, Dr J. Powell, Dr D. H. Simmonds, Professor Wilson Smith, Dr W. W. Umbreit, and Dr D. W. Woolley. Laboratory facilities for various experiments were kindly provided by Professor E. C. Amoroso, Dr E. F. Gale and Dr R. G. McFarlane. London University Central Research Fund enabled purchases of apparatus to be made, and the Rockefeller Research Fund of University College Hospital Medical School provided maintenance grants for D.L.D. and D.S.H.

We are grateful to R. F. Denman and Miss B. C. Knight for technical assistance.

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Preparation of Dextran [^{35}S]Sulphate and Tracer Experiments in the Rabbit

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(*Received* 25 *June* 1954)

The factors influencing the blood anticoagulant activity and toxicity of dextran sulphate have been described in a series of papers (Ricketts, 1952; Ricketts & Walton, 1953*a*, *b*) and clinical trial of a suitable form of dextran sulphate as an anticoagulant drug has been reported (Ricketts *et al.* 1953). The fate of this intravenously injected polysaccharide sulphate in the body is therefore of some interest. Histological staining with the dye toluidine blue shows that the larger molecules of dextran sulphate are taken up into the reticuloendothelial cells of various tissues from which, judging by the diminishing intensity of staining, they slowly disappear. Using this method it has not been possible to show uptake of the smaller molecules and their fate has hitherto been unknown (Walton, 1954).

There are two reports of the use of the radioactive isotope ^{35}S to trace the fate of intravenously injected polysaccharide sulphates. Morrow *et al.* (1952) prepared cellulose [^{35}S]sulphate by reaction of [^{35}S]sulphuric acid with cellulose. The product contained 5–6% S and had an intrinsic viscosity of 1·5–2. Six hours after intravenous injection into cats the radioactivity of various tissues and fluids was measured. On this basis the distribution of this preparation of cellulose sulphate was urine 20–25%, blood 3·6–6·9% and liver 7·2%. Xylan [^{35}S]-sulphate was prepared by Husemann, Hoffman, Lotterle & Widersheim (1952) by treatment of xylan with chloro[^{35}S]sulphonic acid and pyridine. The sulphur content of their xylan sulphates is not quoted but was probably sufficient to confer anticoagulant activity, since this aspect was of interest to the investigators. The fate in rabbits of three preparations of xylan [^{35}S]sulphate with molecular chain lengths averaging 45, 12 and 4 xylose units was deduced from the radioactivity of tissue homogenates. The longer the chain length, the more was stored in tissues, notably liver, and the less was excreted in urine as their figures show:

Average chain length	Percentage of dose found in	
	Liver	Urine
45	21	26
12	9	27
4	0·6	66

It seemed to us that preparation of dextran [^{35}S]-sulphate having a molecular-weight distribution similar to that advocated for a clinical anticoagulant would facilitate experiments settling several important features of the action of heparin-like anticoagulants. The concentration of dextran sulphate in the blood could be correlated with the clotting time. The rate of renal excretion and nature of the material excreted could be studied. The role of renal excretion in terminating the effect of a single injection could be ascertained.

This paper describes the preparation of dextran sulphate of appropriate molecular composition labelled with ^{35}S and some experiments on the fate of this material in the rabbit, in particular the nature and amount of the excretion product. The physiological aspects will be considered in a subsequent paper.

METHODS

Intrinsic viscosity and sulphur content were determined as previously described (Ricketts, 1952).

Determination of dextran sulphate by metachromatic reaction

Dextran sulphate in urine samples was determined through its metachromatic reaction with the dye toluidine blue using the method devised by MacIntosh (1941) for determination of heparin. The metachromatic reaction appears to be the formation of an insoluble purple salt with one or two components of the dye. The principle of MacIntosh's method is that the amount of dye removed from solution is proportional to the amount of a polysaccharide sulphate added within experimentally definable limits which are constant for a given batch of dye. Ricketts & Walton (1954) found that the amount of dye precipitated is proportional to the sulphur content of the dextran sulphate over the range 2–20% and independent of molecular size for dextran sulphate molecules composed of more than four glucose units. In the determinations reported here a Unicam SP600 spectrophotometer was used to determine the amount of toluidine blue remaining in solution, measurements being made at the absorption maximum of 625 mμ. The method was calibrated with a dextran sulphate containing 17·1% S and where the sulphur content of the dextran sulphate being determined differed from this the small proportional correction was introduced. Fractionation experiments (Ricketts & Walton, 1953*a*) show that

laboratory-made dextran sulphates are nearly homogeneous in the distribution of sulphate groups among the molecules.

Though normal rabbit urine often contains metachromatic substances, none was present in samples obtained after the commencement of saline infusion but before injection of dextran sulphate. The samples were also free from protein which was found to interfere with the determination.

Determination of dextran sulphate by radioactivity

In determining dextran sulphate by its radioactivity the technique employed by Rowley, Rowlands & Stuart (1948) for [^{35}S]penicillin was followed. Portions (0·1 ml.) of suitably diluted solutions were dried at 120° on Ni planchets, allowed to cool in a desiccator and counted using a thin end-window Geiger–Müller tube (type EHM 2). It was necessary to correct for the absorption of radiation by other solutes. This absorption decreases with dilution. Dilution is limited by the corresponding reduction in counting rate. A dilution of 1 in 4 with distilled water was found to give a satisfactorily low correction factor without unduly prolonging the counting period.

Paper chromatography of dextran sulphate

Ascending chromatography with 25% (v/v) *n*-propanol in M/15 sodium phosphate buffer (pH 6) and Whatman no. 1 paper, as used by Kerby (1953) for heparin, was found to be useful for sodium dextran sulphate. Reproducibility was improved by equilibration of the paper with the vapour phase for an hour before commencing development of the chromatogram. After development and drying, chromatograms were immersed in a nearly saturated solution of toluidine blue in ethanol for 5 min., then transferred to 40% (v/v) ethanol for a further 5 min. The aqueous ethanol allows the metachromatic reaction to take place without dissolving the dextran sulphate off the paper. Chromatograms were dried by immersion in ethanol and subsequently in warm air. Spots appear purple on a pale blue background. A series of preparations of similar sulphur content (17–19% S) was available from previous work, and it was quickly established that molecular size was the feature determining the R_F value of such preparations. The mechanism of this chromatography is not fully understood, but it seems probable that the same factors are involved as in fractional precipitation of dextran sulphate from aqueous ethanol solution (Ricketts & Walton, 1953*a*). The mobile phase of the chromatogram is analogous to the supernatant solution in fractional precipitation.

Preparation of dextran [^{35}S]sulphate

The molecular-weight distribution advocated for a clinical anticoagulant may be obtained either by fractionation of the dextran or by fractionation of the dextran sulphate itself. The latter procedure would be wasteful of ^{35}S. The dextran fractionation described in a previous paper (Ricketts, 1952) was therefore modified to remove a little more of the high-molecular material.

Partial hydrolysis and fractionation of dextran. To 1 l. of a 6% (w/v) solution of a partial hydrolysate of *Leuconostoc mesenteroides* dextran with intrinsic viscosity 0·32, 100 ml. N-H_2SO_4 were added and the solution was boiled under reflux for 4·5 hr. After cooling, the solution was neutralized with NaOH and the volume was made up to 1200 ml. Acetone (1032 ml.) was added with thorough stirring, and after standing overnight at 21° the syrupy ppt. containing the larger molecules was separated (1·55 g. dextran). The supernatant solution was then cooled at 6° overnight and again the syrupy ppt. was separated and rejected (3·85 g. dextran). More acetone (1968 ml.) was added, the syrup separated at 21°, poured into ethanol, ground to a powder and dried *in vacuo* over P_2O_5, yielding 21 g. dextran of intrinsic viscosity 0·033.

Trial sulphation and tests of molecular composition. A trial sulphation in the same apparatus and on the same scale (0·53 g. dextran) as described below for the radioactive preparation gave a satisfactory yield (91%) of sodium dextran sulphate (1·0 g.) containing 16·4% S. This material was an effective anticoagulant *in vivo*, since an intravenous injection of 10 mg./kg. into a rabbit produced a satisfactory elevation of blood clotting time, i.e. the clotting time was elevated to 3 times the normal value 1 hr. after the injection of this dose. When injected intravenously into two guinea pigs no anaphylactoid response occurred (Ricketts & Walton, 1953*b*). No precipitation of fibrinogen occurred when tested according to Walton (1952). It was therefore assumed that this dextran was of appropriate molecular composition for conversion into radioactive dextran sulphate. A larger scale preparation of dextran sulphate (*S*) from this same dextran was carried out to provide material for control experiments; it contained 17·1% S and had intrinsic viscosity 0·027.

Sulphation with chloro[^{35}S]sulphonic acid. Chlorosulphonic acid (0·7 ml.) was added to pyridine (7·2 ml.) with cooling by solid CO_2 in ethanol, followed by chloro[^{35}S]sulphonic acid (0·06 ml.) containing 1 mc of ^{35}S which was washed with more chlorosulphonic acid (0·4 ml.). The temperature was raised to 60° when all the pyridinium salts dissolved. The above dextran (0·53 g.) was added and stirring was continued at 70–80° for 4 hr. After cooling, water (28 ml.) was added, followed by NaOH (40% w/v) until pyridine separated. To the aqueous phase, ethanol (30 ml.) was added, precipitating sodium dextran sulphate as a syrup together with some sodium sulphate. The syrup was dissolved and dialysed (72 hr.) until the dialysate gave no ppt. with $BaCl_2$. A sample of the final dialysate was set aside for counting and volumes were measured to enable contamination of the product with $^{35}SO_4^{2-}$ to be estimated. The solution (170 ml.) was neutralized, concentrated under reduced pressure and freeze-dried in 8 ampoules giving a total yield of 1·09 g., containing 18·0% S. On this basis 86% of the product had been recovered containing 36% of the available isotope. Under standardized conditions, 20 μg. gave 1567·0 counts/min., or 1 μg. 78·35 counts/min. A sample (0·1 ml.) of the final dialysate gave a count of 57·1/min. and from this it was estimated that 0·114% of the counts was due to contamination of the product with $^{35}SO_4^{2-}$. The background count was 11/min.

Tracer experiments in rabbits

The purpose of these experiments was to find out how much dextran sulphate was excreted and whether the excreted material was identical with that injected. Dextran [^{35}S]sulphate was injected intravenously in doses of 20 and 40 mg./kg. into rabbits during continuous infusion of physiological saline in experiments of the type described in detail by Piper (1947) for heparin. In these experiments urine was collected for six successive 10 min. periods. Dextran sulphate in the urine samples was estimated by the

metachromatic method of MacIntosh (1941) and from the radioactivity as described. After the experiment the rabbits were placed in metabolism cages and urine was collected for 2 or 4 days in an attempt to estimate the total excretion of dextran sulphate.

RESULTS

The results of analysing the urine samples for dextran sulphate are shown in Table 1. The amount excreted in successive 10 min. periods decreases and it was found that the logarithm of the weight of dextran sulphate in each period was linearly related to the time after injection as shown in Fig. 1.

The nature of the dextran sulphate in urine was investigated by paper chromatography. Fig. 2 shows a chromatogram comparing dextran sulphate excreted in urine with dextran sulphate (*S*) identical in molecular composition with that injected. The 'trailing' appearance of spot *S* is believed to indicate the dispersion of molecular weight in this compound, the components of lower R_F value being those of higher molecular weight. The dextran sulphate in urine was characterized by the absence of components with low R_F values as compared with the control. Fig. 3 shows another chromatogram comparing dextran sulphate in urine with control preparations. The control preparation (*OC*) having the same chromatographic behaviour as the dextran sulphate of urine is a mixture of the sulphuric esters of penta- and hexa-saccharides derived from dextran (Ricketts, 1954). Both this control preparation and the dextran sulphate in urine diffused through cellophan membranes. A dialysate of one of the urine samples is included in this chromatogram (Fig. 3). These and similar experiments on the other urine samples showed that the dextran sulphate in the urine up to 1 hr. after injection was not identical with the material injected but appeared to be a fraction of low molecular weight derived from it.

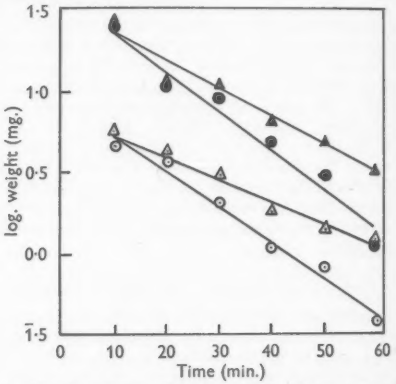

Fig. 1. Renal excretion of dextran [^{35}S]sulphate by rabbits at two dose levels. Dose 40 mg./kg., determinations made by radioactivity (▲—▲) and by metachromatic reaction (●—●). Dose 20 mg./kg., determinations made by radioactivity (△—△) and by metachromatic reaction (⊙—⊙). The logarithm of the corresponding weight of dextran sulphate is plotted against time after injection.

Table 1. *Analysis of rabbit urine samples for dextran sulphate*

Figures in brackets are percentages of dose.

Sample no.	Volume (ml.)	Dextran sulphate (mg.) Metachromatic estimation	Dextran sulphate (mg.) Radioactivity estimation
Rabbit weighing 3·2 kg. given 64 mg. of dextran [^{35}S]sulphate, i.e. 20 mg./kg.			
1	6·0	4·50	5·70
2	4·8	3·49	4·19
3	5·9	2·01	2·97
4	5·2	1·05	1·79
5	4·7	0·79	1·37
6	5·9	0·35	1·17
Total at 1 hr.	—	12·19 (19·0%)	17·39 (27·1%)
Subsequent 48 hr.	162	7·68	5·05
Total at 49 hr.	—	19·87 (31·0%)	22·44 (35·0%)
Calc. total	—	12·50 (19·5%)	19·50 (30·3%)
Rabbit weighing 3·4 kg. given 136 mg. of dextran [^{35}S]sulphate, i.e. 40 mg./kg.			
1	7·2	26·0	29·52
2	5·0	10·95	11·03
3	9·0	8·65	10·75
4	8·6	4·73	6·27
5	8·4	2·78	4·76
6	6·0	1·08	3·03
Total at 1 hr.	—	54·19 (39·9%)	65·36 (48·0%)
Subsequent 96 hr.	—	12·10	7·20
Total at 97 hr.	—	66·29 (48·7%)	72·56 (53·3%)
Calc. total	—	58·50 (43·0%)	65·5 (48·2%)

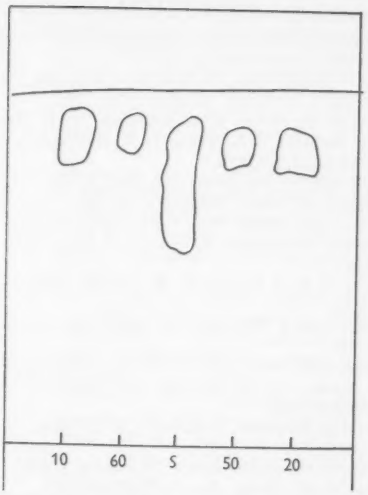

Fig. 2. Paper chromatogram comparing dextran sulphate in urine samples at 10, 20, 50 and 60 min. after injection with a dextran sulphate (*S*) identical in molecular composition with the injected material. Ascending chromatography with 25% (v/v) *n*-propanol in M/15 sodium phosphate buffer (pH 6) and Whatman no. 1 paper.

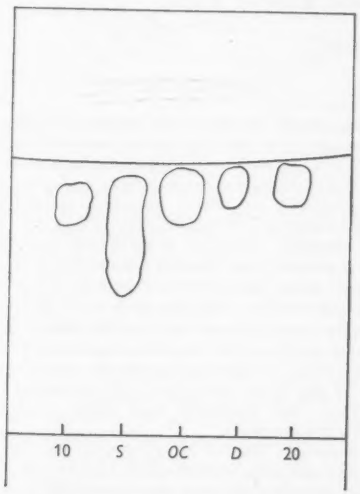

Fig. 3. Paper chromatogram comparing dextran sulphate in urine samples at 10 and 20 min. and also dialysate of the 20 min. sample (*D*) with the dextran sulphate (*S*) and a reference preparation (*OC*) of short chain length. Ascending chromatography with 25% (v/v) *n*-propanol in M/15 sodium phosphate buffer (pH 6) and Whatman no. 1 paper.

DISCUSSION

In considering the results of the tracer experiments, shown in Table 1, the significance of the two methods of estimating dextran sulphate should be borne in mind. The metachromatic estimation measures the number of sulphuric acid ester groups in the urine sample. The radioactivity estimation measures the number of sulphur atoms and may include metabolic products of dextran sulphate.

Since the blank urine samples were free from metachromatic material it is probably justifiable to regard the metachromatic estimates on samples numbered 1 to 6 in Table 1 as measuring only dextran sulphate. The estimate by radioactivity is always the higher of the two.

Fig. 1 shows that whatever may be the true basis of the estimates or the causes of error the excretion follows an exponential decay curve, since the logarithm of the amount excreted in each 10 min. period is linearly related to time after injection.

By regarding the ordinates at 10 min. intervals as a series in geometrical progression an estimate of the total excretion may be obtained as the sum to infinity of the series. This provides the calculated totals of Table 1. It will be seen that the amount found by analysis is slightly greater than the total calculated on the assumption that excretion continues at the uniformly declining rate. (The only discrepancy is the metachromatic estimate at 49 hr. for the rabbit given 20 mg./kg., which is too high. This could have been caused by naturally occurring metachromatic substances.)

Referring again to Fig. 1, the difference in slope of both pairs of lines is significant (lower pair: $t=4{\cdot}37$, $P<0{\cdot}01$; upper pair: $t=2{\cdot}58$, $P<0{\cdot}05$). This implies that the *S* atoms from dextran sulphate are leaving the rabbit more rapidly than they can be accounted for as sulphuric ester groups. The excess sulphur may be in the form of fragments of the dextran sulphate molecule with less than four glucose units or inorganic sulphate so remaining undetected by toluidine blue.

During the first hour after injection the dextran sulphate excreted by the kidney appears to be a low-molecular-weight fraction of that injected. Probably it is excreted so rapidly because it is insufficiently firmly bound to proteins of the clotting system. Dextran sulphate of such short chain length (*OC*) has been shown (Ricketts, 1954) to have relatively little effect upon blood clotting time when injected. Since as much as 48% may ultimately be excreted it is very probable that larger dextran sulphate molecules appear in the urine later on, but their concentration is so low that it is difficult to investigate their molecular weight.

It should be noted that these experiments were carried out with doses larger than a single

therapeutic dose and involving an amount of dextran sulphate corresponding to two or three days of therapy.

SUMMARY

1. Dextran [^{35}S]sulphate with molecular-weight distribution and biological properties similar to that used as a clinical anticoagulant has been prepared.

2. Intravenous injection of dextran sulphate is followed by renal excretion in exponentially decreasing amount. About 30% is eliminated in 2 days rising to 50% at 4 days.

3. Initially the excreted dextran sulphate is a low-molecular-weight component of the injected material.

We should like to thank Professor J. R. Squire for his encouraging interest and helpful suggestions. Thanks are due also to Dr J. P. Bull and Miss A. Fisher for statistical advice. The skilled assistance of Mr M. Hall in the laboratory is gratefully acknowledged.

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Inhibitors and Activators of Brain Hexokinase

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(*Received* 14 *April* 1954)

Inhibitions and activations of hexokinase by specific proteins are of great interest because of their possible connexion with the mechanism of hormonal action. Colowick, Cori & Slein (1947) found that a protein fraction of the anterior pituitary inhibited the hexokinase activity of brain and muscle extracts and that the inhibition was enhanced by adrenocortical extract and counteracted by insulin. Attempts to confirm this result have not always been successful. Abood & Gerard (1951) have reported that a protein in nerve extracts specifically inhibits brain hexokinase; inhibitors of hexokinase have also been found in extracts of spleen (Broh-Kahn & Mirsky, 1947), pancreas (Zakharyn, 1951) and placenta (Gerrard, private communication). Weil-Malherbe & Bone (1951*a–d*) made a detailed study of brain hexokinase and discovered activators of the enzyme in erythrocytes and muscle, and inhibitors and activators in human plasma. Zacco & Sevag (1952) have published a preliminary report on an inhibitor in human plasma. Further experiments with inhibitors and activators of hexokinase are described in this paper.

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EXPERIMENTAL

The experimental arrangements, analytical methods and composition of the medium for the assay of hexokinase activity were as used by Weil-Malherbe & Bone (1951*a*). Hexokinase activity was determined by measuring glucose disappearance (Nelson, 1944). Because of the high adenosinetriphosphatase (ATPase) activity of many of the preparations used, it was usually found convenient to make up the test medium in the following order and final concentrations (M): water; glucose, 0·00185–0·002; $MgCl_2$, 0·0067; $NaHCO_3$ or $KHCO_3$, 0·02; activators or inhibitors; NaF, 0·024. The reaction was started by the addition of adenosine triphosphate (ATP) to give a final concentration of 0·0033–0·005 M. Incubations were usually carried out at 30° for 15–20 min. in an atmosphere of N_2 containing 5% (by vol.) CO_2. Any deviations from these conditions are mentioned in the text.

Brain extracts. Most of the experiments were carried out with an extract prepared by double extraction of rat brain at 0° with glass-distilled water and centrifuging in an angle centrifuge at 3500 rev./min. for 5 min. (Weil-Malherbe & Bone, 1951*a*). This preparation will be referred to as 'standard brain extract'. Some brain extracts were fractionated by centrifuging at 20 000 *g* for 20 min. in a refrigerated centrifuge, a procedure which practically eliminates particulate constituents other than microsomes. The supernatants will be referred to as high-speed extracts.

Plasma inhibitor. Blood samples (5–10 ml.) were obtained by venipuncture and delivered into bottles containing solid NaF as anticoagulant to give a final concentration of 0·3% (w/v). The NaF was dissolved by gently inverting the bottle for a few minutes and the plasma separated by centrifuging. The plasma was either used at once or exhaustively dialysed at 3° against running glass-distilled water.

Nerve suspensions. Sciatic and brachial nerves of male white rats were ground with sand and glass-distilled water at 0°. The mixture was centrifuged lightly to remove sand and tissue debris and the residue extracted once more with water. The aqueous suspension (1:20) was used without further purification.

Placental extracts. Aqueous extracts of human placenta (1:20) were made available through the courtesy of Dr J. W. Gerrard. The extracts were frozen by solid CO_2 and despatched in boxes containing solid CO_2. They were still frozen when received.

Erythrocyte lysates and rat-muscle extracts. Haemolysates and muscle extracts were prepared according to Weil-Malherbe & Bone (1951*b*). The muscle extracts were dialysed against running glass-distilled water at 3° and used without further purification.

In experiments involving activations or inhibitions, deviations $\geqslant 17\%$ from the control experiment were regarded as significant. Weil-Malherbe & Bone (1951*d*), using identical experimental methods, established that deviations $\geqslant 17\%$ were statistically significant at the 1% level.

RESULTS

Inhibitor in plasma

Occurrence. Significant inhibitions were found in four out of thirty-eight non-dialysed samples of plasma, i.e. in 10·5% of cases. Zacco & Sevag (1952) found inhibitions in 14% of their cases and Weil-Malherbe & Bone (1951*d*) in 13% of a series which included about 400 samples. In agreement with observations by Zacco & Sevag (1952), it was found that dialysis of plasma against glass-distilled water enhanced and stabilized the inhibitor activity. When the standard test medium contained 1–1·5 ml. of plasma, twenty-one out of twenty-four samples of dialysed plasma significantly inhibited brain hexokinase activity, in fourteen cases by over 30%. When blood samples were drawn at 30 min. intervals during a glucose-tolerance test, and the plasma was separated, dialysed and tested, the inhibitions observed did not vary significantly from sample to sample, nor were there any marked differences in inhibition among plasma samples from the same donor taken at intervals of several months.

Properties of the dialysed plasma inhibitor. The inhibitor was non-dialysable and relatively heat stable. The inhibition was not counteracted by insulin or reduced glutathione (GSH) (Table 1). The relation between inhibition and inhibitor concentration was non-linear; increasing the volume of dialysed plasma beyond 1·5 ml./3 ml. reaction mixture did not appear to lead to further increase in inhibition (Fig. 1). Inhibition was maximal from the start of the reaction (Fig. 2); preincubation of the brain extract with plasma for 20 min. at 30° had no effect on the inhibition (Table 1). The activity of samples of non-dialysed plasma showing inhibition disappeared so quickly even at 3° that a systematic investigation of their properties was not practicable.

Specificity. Inhibitions were most consistently obtained with brain hexokinase and were about the same for standard and high-speed extracts (Table 2). Yeast hexokinase and brain phosphohexokinase were also inhibited, apparently to a somewhat smaller extent. Inhibition was also observed with fructose as substrate (Table 3).

Reversibility. It was found that the inhibition by dialysed plasma was not counteracted by salt addition. When KCl was added to plasma, which had been exhaustively dialysed against glass-distilled water, to give a final concentration of 0·67 M, the inhibition was not affected (Table 4).

Table 1. *Inhibition of brain hexokinase by dialysed plasma*

Standard brain extract, 0·3 ml.; dialysed plasma, 1·5 ml.; ATP, 15 μmoles; total vol., 3 ml. Other components in standard concentration.

Additions	Hexokinase activity (control = 100)
Plasma	65
Plasma + 0·5 mg. insulin	63
Plasma heated for 3 min. at 100°	65
Plasma heated for 10 min. at 55°	73
Plasma + 0·003 M GSH	63
Plasma preincubated with enzyme for 20 min. at 30°	66

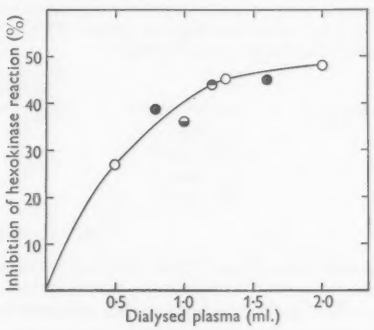

Fig. 1. Effect of plasma concentration on the activity of rat-brain hexokinase. Brain extract, 0·3 ml.; ATP, 15 μmoles; total vol., 3 ml.; other constituents in standard concentration. Each symbol represents a different standard brain extract.

Mode of action. The inhibition by plasma is not due to ATPase or phosphatase activity. When the magnesium and ATP concentrations were increased fourfold the inhibition was not relieved. The Mg:ATP ratio was kept constant, as Hers (1952) has shown that the activity of brain hexokinase is reduced at Mg:ATP ratios below 1. Furthermore, when ATP was estimated at the end of the incubation by determining the acid-labile phosphate, corrected for hexose diphosphate (HDP) hydrolysis (Weil-Malherbe & Bone, 1951*a*), the ATP concentration in the presence of plasma was actually higher than in the control (Table 5). The inhibition was also unaffected when the glucose concentration was reduced by a factor of 4 and that of brain extract to 0·1 ml./3 ml. reaction mixture. At this, the lowest substrate concentration at which the inhibition could be accurately measured, the reaction was still of zero order with respect to glucose.

Experiments in which the inhibition of hexokinase is determined by measuring glucose disappearance are vitiated if reducing substances are produced in the reaction mixture (Stadie, Haugaard & Hills, 1950). This source of error may be excluded, as no reducing substances were produced when brain extracts were incubated for 20 min. at 30° in reaction mixtures containing inhibitor but no ATP. The results reported here suggest that the inhibitor acts directly on the enzyme.

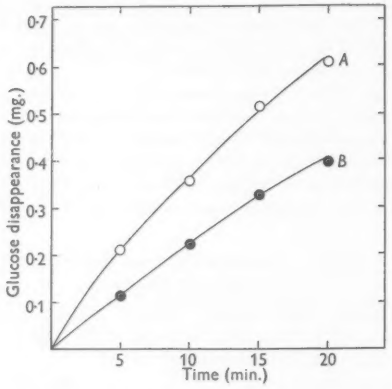

Fig. 2. Disappearance of glucose from rat-brain extract in the presence and absence of plasma inhibitor. Brain extract, 0·3 ml.; glucose, 6·66 μmoles.; ATP, 15 μmoles; total vol., 3 ml. Curve *A*, brain extract; curve *B*, brain extract + 2 ml. dialysed plasma.

Table 2. *The effect of dialysed plasma on standard and high-speed extracts of brain*

Standard brain extract, 0·3 ml., or high-speed extract, 0·6 ml.; ATP, 15 μmoles; dialysed plasma, 2·0 ml. Other components in standard concentration.

Expt. no.	Type of extract	Hexokinase activity (control = 100)
1	Standard	79
	High-speed	80
2	Standard	78
	High-speed	78

Table 4. *The effect of* KCl *on the inhibition of brain hexokinase by dialysed plasma*

Brain extract, 0·3 ml.; ATP, 15 μmoles; dialysed plasma, 1·7 ml. Other components in standard concentration.

Expt. no.	Additions	Hexokinase activity (control = 100)
1	0·67 M-KCl	64
	Plasma	76
	Plasma + 0·67 M-KCl	49 (77*)
2	0·67 M-KCl	62
	Plasma	76
	Plasma + 0·67 M-KCl	48 (77*)

* Calculated for KCl-containing medium as control.

Table 5. *ATP concentration in brain extracts after 20 min. incubation*

Standard brain extract, 0·3 ml.; plasma, 1·5 ml. in Expt. 1, 1·8 ml. in Expt. 2; ATP, 15 μmoles; total vol. 3 ml.; other components in standard concentration.

Expt. no.	Additions	ATP found (μmoles)	Inhibition of hexokinase reaction (%)
1	Nil	6·4	—
	Plasma	7·0	45
2	Nil	7·1	—
	Plasma	8·6	47

Table 3. *Specificity of plasma inhibitor*

Standard brain extract, 0·3 ml., or yeast hexokinase, 0·4 mg.; dialysed plasma, 1·5 ml.; ATP, 15 μmoles; other components in standard concentration; hexose monophosphate (HMP) in phosphohexokinase experiment, 15 μmoles; total vol., 3 ml. Phosphohexokinase activity measured by HDP formed.

No. of measurements	Enzyme	Substrate	Mean inhibition of enzyme activity (%)
5	Brain hexokinase	Glucose	38
5	Brain phosphohexokinase	HMP	22
2	Brain hexokinase	Fructose	20
2	Yeast hexokinase	Glucose	17

Inhibitor in nerve and placenta

The necessity for showing, as in the preceding section, that inhibition of hexokinase is not caused by ATPase or phosphatase activity is well illustrated by the following experiments with nerve suspensions and placental extracts.

Suspensions from the nerves of twenty-two rats were tested. Of these, seven significantly inhibited brain hexokinase, but the effect per mg. of nerve was only 0·5–0·3 as great as that reported by Abood & Gerard (1951). The inhibitor lost most of its activity within a few hours, even at 3°. It could not be stabilized by dialysis against glass-distilled water. The inhibition by nerve suspensions could be counteracted by excess ATP (Fig. 3). This suggests that the inhibition is caused by ATPase activity. In fact, during incubation for 20 min. in the presence of fluoride, active preparations hydrolysed over 25% of the ATP in the reaction mixture, and ATPase activity of this order was always present when inhibition was observed. Phosphate formation from hexose monophosphate (HMP) and HDP was about 20 times smaller. Furthermore, it was found that incubation at 37° for 40 min., the experimental conditions of Abood & Gerard (1951), required a minimum ATP concentration for maximum activity of brain hexokinase of 0·006M, while these authors used 0·003M-ATP. At low initial ATP concentrations hydrolysis of ATP by ATPase will have an inhibitory effect on glucose phosphorylation and will also favour the accumulation of inhibitory HMP. The inhibitions observed by Abood & Gerard (1951) can be fully accounted for on this basis.

The inhibition of brain hexokinase by placental extracts proceeds by a similar mechanism. Inhibitions were consistently obtained both with crude extracts and with an ammonium sulphate fraction precipitated between 0·25 and 0·33 saturation. Again the inhibitions were reversible by excess ATP (Fig. 4). The placental extracts differed from the nerve suspensions in their ability to hydrolyse HMP and HDP as well as ATP.

Activators of hexokinase

Apparent activation of the hexokinase reaction by phosphohexokinase. In a recent paper on brain hexokinase, Crane & Sols (1953) found that the bulk of the enzyme sediments at 3500 *g*. The rate of glucose utilization by the sediment decreased with time as the phosphohexokinase (phosphohexose isomerase + phosphofructokinase) concentration in the mixture was insufficient to prevent the accumulation of inhibitory glucose 6-phosphate. The addition of phosphohexokinase led to a significant increase in the rate of glucose utilization.

Apparent activations of this type were observed in some cases with brain preparations which contained a much higher proportion of particulate hexokinase than the standard extract (Table 6). In the standard extract about 0·5–0·7 of the hexokinase activity was associated with particulate matter. The proportion of hexokinase left in the supernatant after centrifuging at 20000 *g* was thus considerably greater than that found by Crane & Sols (1953). This was also the case when isotonic

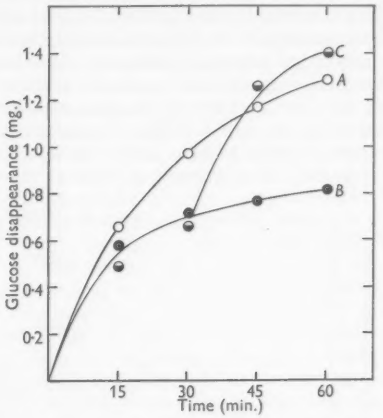

Fig. 3. Disappearance of glucose from rat-brain extract in the presence and absence of nerve suspension. Brain extract, 0·25 ml.; glucose, 7·8 μmoles; ATP, 7·5 μmoles; other constituents in standard concentration; total vol., 3 ml. Curve *A*, brain extract; curve *B*, brain extract + nerve suspension from 30 mg. nerve; curve *C*, same as curve *B*, with 5 μmoles ATP added at 30 min.

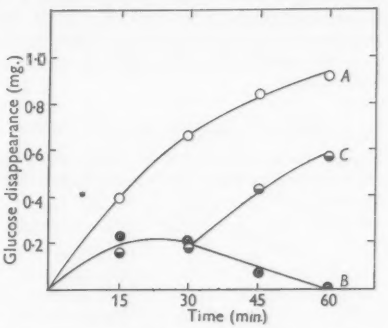

Fig. 4. Disappearance of glucose from rat-brain extract in the presence and absence of placental extract. Brain extract, 0·2 ml.; glucose, 5·55 μmoles.; ATP, 10 μmoles; other constituents in standard concentration; total vol., 3 ml. Curve *A*, brain extract; curve *B*, brain extract + placental extract (10 mg. protein); curve *C*, same as curve *B*, with 10 μmoles ATP added at 30 min.

mannitol was used as suspending medium. The difference might be due to the use of a tissue grinder of closer fit. The utilization of HMP by the standard extract was about 2–3 times as great as that of glucose for a substrate concentration of 0·002 M.

Activator in erythrocytes and muscle. The activation of brain hexokinase by extracts of erythrocytes and muscle (Weil-Malherbe & Bone, 1951*b*, *c*) cannot be explained by the HMP–phosphohexokinase mechanism. This was shown kinetically by carrying out an experiment at two concentrations of brain hexokinase. At the lower concentration it will take longer before an inhibitory concentration of HMP is produced, and if the activation is due to the removal of HMP by phosphohexokinase it should be greater at the higher concentration of hexokinase (Crane & Sols, 1953). It was found, however, that the reaction was linear at two concentrations of brain hexokinase, namely 0·3 and 1·0 ml. of a high-speed extract in a total volume of 3 ml., and the activation was the same in both cases (Fig. 5). This result, together with the findings of Weil-Malherbe & Bone (1951*b*, *c*), that haemolysates and muscle extracts in which phosphohexokinase activity had been destroyed by heat were still able to activate brain hexokinase, provide conclusive evidence for the non-identity of the activator and phosphohexokinase.

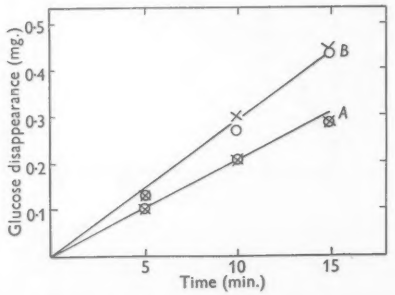

Fig. 5. Activating effect of dialysed rat-muscle extract on brain hexokinase at two concentrations of brain extract. High-speed extract, 0·3 ml. (×) and 1·0 ml. (○) in 3 ml. total volume; muscle extract, 1·0 ml. Curve *A*, brain extract; curve *B*, brain extract + muscle extract. Glucose disappearance expressed in mg./ml. of brain extract used. Both curves corrected for the hexokinase activity of the muscle extract.

Effect of salt concentration on the activation of brain hexokinase. When standard brain extract was incubated in the presence of 0·67 M-KCl or 0·003 M-$CaCl_2$ and activator, hexokinase activity was somewhat reduced but the activation was completely abolished (Table 7).

Effect of brain preparation on activation. One muscle extract was tested with a series of brain extracts. 1 ml. of the muscle extract formed 0·44 μmole of HDP from 0·005 M-HMP in 20 min. under the conditions of the standard test. The phosphohexokinase activity of 1 ml. of brain extract was 20–30 times greater. Increasing the volume of muscle extract added beyond 0·3 ml. in 3 ml. reaction mixture did not lead to further increase in activation. It was found in these experiments that the extent of the activation varied with different brain extracts. Furthermore, it was sometimes the high-speed extract and sometimes the standard extract which was more susceptible to activation (Table 8). This strongly suggests that the activation depends on a factor in the brain preparation.

DISCUSSION

In the experiments with nerve and placental extracts the observed inhibitions could not be separated from the ATPase or phosphatase activity of the preparations. On the other hand, the data of this paper confirm the existence of an inhibitor of

Table 6. *Hexokinase activity of brain preparations separated by fractional centrifuging*

Rat brain was macerated in 6 ml. 5·75% mannitol, centrifuged 5 min. at low speed and 20 min. at high speed. Both sediments were washed once with mannitol, recentrifuged, and made up to original volume; 0·2 ml. was added to standard test system.

Expt. no.	Fraction	Centrifugal force (*g*)	Disappearance of glucose (mg.)	Apparent activation*
1	*A* (Sediment)	1 500	0·295	—
	B (Sediment)	20 000	0·137	—
	C (Supernatant)	20 000	0·096	—
	B + *C*	—	0·268	115
	A + *C*	—	0·844	216
2	*A* (Sediment)	1 200	0·263	—
	B (Sediment)	20 000	0·251	—
	C (Supernatant)	20 000	0·117	—
	A + *B* + *C*	—	0·617	98

* Apparent activation = ratio of activity of combined fractions to sum of activities of individual fractions tested separately, × 100.

Table 7. *The effect of electrolyte concentration on the activation of brain hexokinase by haemolysates*

Haemolysate heated 5 min. at 80°; 1 ml. haemolysate added to standard test.

Expt. no.	Additions	Hexokinase activity (control = 100)
1	0·85 M-KCl	57
	Haemolysate	140
	Haemolysate + 0·85 M-KCl	54
2	0·67 M-KCl	72
	Haemolysate	146
	Haemolysate + 0·67 M-KCl	63
3	0·003 M-$CaCl_2$	59
	Haemolysate	129
	Haemolysate + 0·003 M-$CaCl_2$	63

Table 8. *The effect of a rat-muscle preparation on a series of brain extracts*

Standard brain extract, 0·3 ml.; high-speed extract, 1·0 ml.; ATP, 15 μmoles. Other components in standard concentration.

Brain extract	Type of extract	Muscle extract (ml.)	Hexokinase activity of brain extract* (control = 100)
1	Standard	0·3	160
2	Standard	0·6	131
	High-speed	0·6	115
3	Standard	0·6	159
	High-speed	0·6	153
4	Standard	1·0	125
	High-speed	1·0	152
5	Standard	1·0	127
	High-speed	1·0	154
	Standard	0·7	130
	High-speed	0·7	159

* Corrected for the hexokinase activity of the rat-muscle extract.

hexokinase in plasma and provide conclusive evidence that the inhibition is not due to phosphatase or ATPase activity. With dialysed plasma, inhibitions are observed in a high proportion of cases. The mechanism by which dialysis enhances and stabilizes the inhibition is not clear. There can be little doubt that the inhibitor in dialysed plasma is identical with that described by Zacco & Sevag (1952). There are, however, important differences in the properties of the inhibitor in dialysed plasma and the inhibitor in fresh plasma described by Weil-Malherbe & Bone (1951*d*). The latter is less stable, occurs less regularly, and the magnitude of the inhibition shows much greater fluctuations over the 3 hr. period of a glucose-tolerance test. Weil-Malherbe & Bone (1951*d*) also found that the inhibitions produced by fresh plasma could be partially relieved by insulin, which was found to have no effect on the inhibition by dialysed plasma.

There is evidence from the work of Christensen, Plimpton & Ball (1949) that an inhibitor of erythrocyte hexokinase is present in the plasma of normal, alloxan-diabetic and hypophysectomized rats. Very recently, Bornstein & Park (1953) and Bornstein (1953) have reported that the β-lipoprotein fraction of the serum of alloxan-diabetic rats inhibits the glucose uptake of rat diaphragm. The inhibitor, which is antagonized by insulin, appears to act at the hexokinase level. This inhibitor was not detected in the serum of normal rats or the serum of diabetic rats which had in addition been hypophysectomized or adrenalectomized. There exists, therefore, the possibility that human plasma and rat serum contain more than one inhibitor of hexokinase.

The significant feature of the experiments with muscle extracts and haemolysates is that the observed activations cannot be accounted for by phosphohexokinase activity. In this connexion it may be mentioned that an activator of hexokinase, free from hexokinase and phosphohexokinase activity, has recently been prepared from ox brain (Weil-Malherbe, private communication). The fact that the activation is abolished by salt is also difficult to reconcile with the view that phosphohexokinase is responsible for the activation. Furthermore, it has been shown that the activation depends on the hexokinase preparation as well as the activator. It appears that the simplest explanation of the experiments with activators is that brain preparations contain an inhibitor. Evidence of a similar kind is provided by the observations of Weil-Malherbe & Bone (1951*d*) that some samples of fresh plasma gave highly significant activations with some and highly significant inhibitions with other preparations of hexokinase. It seems plausible therefore, to visualize the activation process in terms of a competition of specific proteins for an inhibitor associated with hexokinase.

SUMMARY

1. The presence in plasma of an inhibitor of hexokinase has been confirmed. Dialysis against glass-distilled water stabilizes and enhances the inhibition. The inhibitor is non-dialysable, fairly heat stable, and not counteracted by insulin. Brain hexokinase with glucose as substrate is most susceptible to inhibition, but the phosphorylations of fructose by brain hexokinase, of hexose monophosphate by brain phosphohexokinase, and of glucose by yeast hexokinase are also inhibited.

2. The inhibition by dialysed plasma is not due to adenosinetriphosphatase activity and is not affected by a fourfold change in the concentration of magnesium, adenosine triphosphate or glucose. It is

suggested that the inhibitor acts directly on the enzyme.

3. The inhibitions of brain hexokinase by nerve and placenta is fully accounted for by their adenosine triphosphatase and phosphatase activities.

4. The activation of hexokinase by proteins in muscle and haemolysates has been confirmed. The phenomenon cannot be adequately explained by the removal of hexose monophosphate by phosphohexokinase. The activation is abolished by 0·67M-KCl and 0·003M-$CaCl_2$. Evidence is given that the activation depends on the hexokinase preparation.

5. The relation of the plasma inhibitor to similar factors described in the literature is discussed. It is tentatively suggested that the activation process involves the competition of specific proteins in the activator preparation for an inhibitor associated with hexokinase.

The author is greatly indebted to Dr H. Weil-Malherbe for much encouragement and advice throughout the course of this work. He also wishes to thank Dr J. W. Gerrard for the placental extracts and the Medical Research Council for a grant.

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Metabolism of Dehydroascorbic Acid and the Effect of Adrenocorticotrophic Hormone and Cortisone

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(*Received* 15 *March* 1954)

In studies designed to elucidate the problem of the fate of dehydroascorbic acid in guinea pigs, Gould & Shwachman (1943) found that oral or injected solutions of ascorbic acid, which had undergone oxidation by treatment with iodine, exerted an antiscorbutic effect. Penney & Zilva (1943) and Damron, Monier & Roe (1952), using solutions of ascorbic acid oxidized by potassium iodate and bromine respectively, found that single injections of dehydroascorbic acid were partly reduced to ascorbic acid and partly oxidized to diketogulonic acid.

To human subjects Johnson & Zilva (1934) gave oral solutions of dehydroascorbic acid prepared by iodine and hydrogen peroxide oxidation of lemon juice; Todhunter, McMillan & Ehmke (1950) administered orally solutions containing dehydroascorbic acid prepared by charcoal treatment of orange juice, while de Ritter, Cohen & Rubin (1951) gave dehydroascorbic acid or its methanolate by mouth. From the effects on plasma concentration or urinary output or both, these investigators concluded that oral dehydroascorbic acid has the same effect as ascorbic acid. We are not aware of any experiments in which dehydroascorbic acid has been injected into human subjects.

Additional interest in dehydroascorbic acid was stimulated by the suggestion of Long, Miles & Perry (1951) based on the modification of tuberculin sensitivity in guinea pigs, that adrenocorticotrophic hormone and cortisone exert their beneficial actions by facilitating conversion of ascorbic acid into dehydroascorbic acid.

Therefore, when relatively pure preparations of dehydroascorbic acid became available, it was decided to study the degree of its reduction to ascorbic acid when administered orally or by injection to guinea pigs and to human subjects and whether adrenocorticotrophic hormone (ACTH) or cortisone would inhibit this reduction. The possible effects of adrenocorticotrophic hormone and cortisone favouring the oxidation of injected ascorbic acid were also studied.

* Holden Research Fellow.

MATERIALS AND METHODS

Dehydroascorbic acid (sample *A*, Boots Pure Drug Co. Ltd.). This was supplied as a yellow powder containing 80% of the acid, 0·8% ascorbic acid, no diketogulonic acid and unknown impurities. This sample was considered unsuitable for injection into human subjects. It was used for injection into some guinea pigs and for oral administration to two human volunteers (subjects 1 and 2).

Dehydroascorbic acid methanolate (sample *B*, Roche Products Ltd.). This was prepared by the method of Pecherer (1951) and was used for other experiments. The colourless crystals were readily soluble in water and promptly dissociated to dehydroascorbic acid and methanol. No adverse effects were observed from the small amount of methanol given (which was 15% of the dose of dehydroascorbic acid). All doses of dehydroascorbic acid are expressed in mg. of the acid. When fresh, dehydroascorbic acid methanolate was 98% pure but, on storage for approximately 2 years, the purity decreased to 66%; at this stage it contained 4% diketogulonic acid. The fresh material was used for injection into human subjects and the stored material for injection into some guinea pigs with due allowance for the decrease in dehydroascorbic content; the quantity of diketogulonic acid injected was too small to influence our results. (A possible exception is the urinary excretion of R & K material (for definition see this page) by guinea pig *Z*, which was injected with large quantities of this sample, containing approximately 6 mg./day of diketogulonic acid.) The methanolate contained no ascorbic acid. Intramuscular injections into humans of this sample of dehydroascorbic acid were so painful that the solutions were mixed with 4% (w/v) procaine (0·25 vol.) immediately before injection; assay of the stability of dehydroascorbic acid in procaine *in vitro* showed a loss of 2% in 1 hr. Where ascorbic acid was injected intramuscularly for control periods it was mixed with the same proportion of 4% procaine.

Ascorbic acid. This was administered to human subjects as tablets by the oral and in saline solution by intravenous or intramuscular routes; for guinea pigs it was dissolved in water before injection. Sodium ascorbate was prepared by almost neutralizing ascorbic acid with 9% (w/v) NaOH or 1·85% (w/v) $NaHCO_3$ immediately before administration, and was used in some investigations because preliminary experiments had suggested that it caused less tissue necrosis than the free acid and might therefore be more regularly absorbed; subsequently it became clear that the rates of absorption were indistinguishable.

Hormone preparations. Lyophilized ACTH (Armour Laboratories, Hampden Park, Eastbourne, Sussex; Organon Laboratories Ltd., Brettenham House, London, W.C. 2) or ACTHAR gel (Armour Laboratories) were used as indicated; dosage is expressed in international units. Cortisone was given as a suspension of its acetate (Merck and Co. Inc., Rahway, New Jersey, U.S.A.); dosages are expressed in mg. of the acetate.

Chemical determination of ascorbic acid, dehydroascorbic acid and diketogulonic acid. Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol (dye); since this titration measures unidentified material present in urine in addition to ascorbic acid, the formaldehyde correction procedure (McSwiney, Clayton & Prunty, 1954) was applied to all human urines except those of subjects 1 and 2. Application of this formaldehyde procedure to several hundred samples of guinea pig urine showed a nearly constant output of 0·3–0·6 mg./day of impurities (in terms of ascorbic acid), and the results with this method, in the case of guinea pigs, have been omitted in this report. Ascorbic acid proved stable in liver filtrates; in adrenal filtrate, however, there was a disappearance of approximately 10% in 2 hr. and adrenal filtrates were therefore titrated with dye within a few minutes of preparation.

Dehydroascorbic acid in biological material was determined by H_2S treatment followed by titration with dye. The dinitrophenylhydrazine method of Roe & Kuether (1943), as modified by Geschwind, Williams & Li (1951) was used for the determination of ascorbic + dehydroascorbic + diketogulonic acids; since this method may also measure unidentified impurities (Penney & Zilva, 1945), the group of substances measured by this technique will be referred to as R & K material and expressed as ascorbic acid.

Assays of purity of dehydroascorbic acid. Assays were carried out by both the dinitrophenylhydrazine method, and by reduction with H_2S at pH 3·5 followed by titration with iodine (Kenyon & Munro, 1948) or dye.

Sterilization of dehydroascorbic acid solutions. For injection into human subjects solutions were sterilized by filtration through sintered glass of porosity 3. Assays of solutions before and after filtration showed no change in dehydroascorbic acid concentration and the solutions were sterile on culture.

Treatment of guinea pigs and analysis of tissues. Guinea pigs were brought into the laboratory several days before use and were maintained on a diet free from ascorbic acid (Harris & Ray, 1932: scorbutic diet) supplemented as necessary by cabbage or by synthetic ascorbic acid, sodium ascorbate or dehydroascorbic acid. The last three were administered subcutaneously in 0·2–0·5 ml. of solution twice daily unless otherwise stated.

Tissue analysis. Preliminary experiments indicated that killing by stunning and bleeding gave more regular results than death by decapitation, ether, Nembutal (Abbott Laboratories Ltd.) or coal gas. They also showed that rapidity of technique was essential at all stages. When each guinea pig was killed the adrenal glands and liver were dissected out rapidly, placed in tared vessels containing sand and 10·0 ml. or 50·0 ml. respectively of 5% (w/v) metaphosphoric acid and immediately ground to a pulp. Solutions were filtered through two layers of Whatman no. 42 paper. Portions of filtrate were titrated with dye and other portions were placed in 6% (w/v) trichloroacetic acid and stored at −15° for the determination of R & K material. The process of killing, dissection and grinding was completed in 4 min.

RESULTS

Antiscorbutic effect of dehydroascorbic acid in guinea pigs

Groups of male guinea pigs each weighing 300–350 g. were kept on the scorbutic diet supplemented with cabbage for 21 days. The cabbage was then stopped, and when the body weights began to fall during a depletion period of 14–20 days, treatment with dehydroascorbic acid was begun as shown in Table 1 (groups 2–5). Treatment resulted in a gain in weight for a period varying from as little as 4 days

Table 1. *Antiscorbutic properties of dehydroascorbic acid in guinea pigs*

Dehydroascorbic acid sample *A* was used for groups 2, 3, 6 and 7; sample *B* for groups 4 and 5. (M) = administered by mouth, (I) = by injection.

Group no.	No. of animals	Period of depletion before administering supplement (days)	Daily dose of supplement (mg.)	Period of survival after withdrawal of cabbage (days)
1	14	—	None	Mean 28·6 ± 1·0 (S.E.)
			Dehydroascorbic acid	
2	4	14–18	20 (M); 60 after 19 days	25, 87, 134, 180
3	4	14–18	20 (I); 60 after 19 days	26, 39, 96, 136
4	3	20	60 (M)	52, 60, 103
5	3	20	60 (I)	10, 25, 59
6	4	None	2 (M)	4, 13, 37, 69
7	4	None	2 (I)	56, 77. Two used for another expt. after 49 days
			Ascorbic acid	
8	3	14–18	20 (M); 60 after 19 days	Over 4 months
9	4	None	2 (M)	Over 4 months
10	4	None	2 (I)	Over 4 months

to as much as 4 months, though all animals ultimately lost weight and died. Groups 6 and 7 were not depleted before treatment with 2 mg. daily of dehydroascorbic acid. Animals receiving this small dose by mouth did not gain weight at all, but injected animals gained for 28–44 days. The control group (group 1) received no treatment and died of typical acute scurvy; control animals receiving a supplement of ascorbic acid (groups 8–10) continued to gain in weight. All animals treated with dehydroascorbic acid ultimately died, though the majority survived considerably longer than if they had received no treatment at all.

With either preparation of dehydroascorbic acid, 2 to 3 weeks before death the guinea pigs began to lose their fur, especially over the terminal phalanges of the fore and hind feet, and the remaining hair became ruffled. In all except one guinea pig, the digits became flexed, and in half the animals respiratory difficulty developed a day or two before death, and there was partial or complete paralysis of the hind legs. On post mortem, even in the animals which died within the time limit of the controls, there was no evidence of scurvy; no haemorrhages were present, and adrenal R & K material in four animals in groups 2 and 3 varied between 11·0 and 44·6 mg./100 g. tissue. The most striking feature was gross fatty degeneration in all the livers except one of those treated with 60 mg. dehydroascorbic acid (sample *A*) and two of those treated with dehydroascorbic acid methanolate. It was especially marked towards the centres of the lobules. Macroscopically, no other pathological changes were evident. Section of the ends of the bones at the joints showed that the orderly arrangement of the cells at the epiphyses was maintained, though the matrix appeared somewhat denser than normal. The marrow contained more fat cells than normal. No changes were apparent on staining the pancreas with haematoxylin and eosin.

The fate of administered dehydroascorbic acid in guinea pig tissues

Measurement of degree of oxidation of ascorbic acid. The amounts of dehydroascorbic acid in guinea pig tissues were so small that they could not be determined accurately, if indeed this substance is present at all. Therefore the ratio of ascorbic acid/R & K material was used as a measure of the degree of oxidation of ascorbic acid. When ascorbic acid concentrations are low (e.g. below 20 mg./100 g. of adrenal tissue), both methods became inaccurate and the ratio is doubly unreliable. Concentrations in liver filtrates were sufficiently high for accurate measurement, except in scorbutic animals.

All guinea pigs used for tissue analyses weighed 250–300 g. Each time a series of experimental guinea pigs was killed, three or four controls which had received cabbage daily were killed and their adrenal ascorbic acid was determined as a check on technique. In thirty-two such animals the ratio of ascorbic acid/R & K material was 0·91 ± 0·026.

Dose equivalence. After a period of depletion to ensure low tissue ascorbic acid concentrations, guinea pigs were injected with varying doses of dehydroascorbic acid or ascorbic acid for 3 days, killed on the 4th day, and the adrenal and liver ascorbic acid were determined. The results are shown in

Table 2. Treatment with both ascorbic and dehydroascorbic acids raised the tissue concentrations of ascorbic acid and R & K material. However, where the doses of ascorbic acid and dehydroascorbic acid were equal, these concentrations were lower in animals treated with dehydroascorbic acid; where the dose of dehydroascorbic acid was twice the dose of ascorbic acid, the tissue levels were similar and there was no significant difference in the ratios of ascorbic acid/R & K material.

Effects of ACTH and cortisone

Long-term experiments. Groups of guinea pigs were depleted for 12 days and then injected with dehydroascorbic acid or ascorbic acid for 3 days with or without administration of cortisone acetate or ACTH. The administration of ACTH and cortisone acetate was begun 24 hr. before that of ascorbic or dehydroascorbic acid. The results are shown in Table 3. No condition in this experiment favoured either the formation or persistence of dehydroascorbic acid, since none of the ratios of ascorbic acid/R & K material were significantly changed in adrenals or liver as a result of treatment; for significance with these numbers of degrees of freedom, t must be greater than 2. In addition, the total amount of R & K material accumulating in the livers following treatment with ascorbic or dehydroascorbic acid was not significantly changed.

Short-term experiments. For 3–5 days before ACTH treatment, healthy guinea pigs were placed on the scorbutic diet supplemented by 100 g. cabbage daily. On the day of the experiment no cabbage was given. Groups 1–7 (Table 4) received no ascorbic acid by injection. Groups 3–7 received an intraperitoneal injection of ACTH and were killed at intervals up to 4 hr. thereafter. Groups 8–16 were given one subcutaneous injection of ascorbic acid or sodium ascorbate combined, in groups 11, 12, 15 and 16, with a simultaneous subcutaneous injection of ACTH. Animals were killed 2 and 6 hr. later. Detailed results, shown in Table 4, indicate that there was no significant change in the ratio of ascorbic acid/R & K material as a result of ACTH injection. There is a significant fall in both ascorbic acid and R & K material ($P < 0.05$). No change occurred in the ratio in the liver of animals in groups 6 and 7.

Urinary excretion products of dehydroascorbic acid. Guinea pigs used weighed 470–600 g. Guinea pig *G* (Table 5) was maintained on the scorbutic diet supplemented by ascorbic acid for days 1 to 7. On days 8 to 16, ascorbic acid was replaced by the same dosage of dehydroascorbic acid. On days 17 and 18 the same dose of dehydroascorbic acid was injected subcutaneously and the animal died during day 18. Results show that this large oral dose of ascorbic acid gave minimal excretion of ascorbic acid, dehydroascorbic acid and R & K material in the urine; with dehydroascorbic acid given orally the excretion of all three increased but still remained only a small fraction of the total dose; with injected dehydroascorbic acid, the urinary excretion of ascorbic acid and R & K material showed a more marked increase.

Two guinea pigs, *B* and *C*, on the scorbutic diet were given courses of ascorbic acid and dehydroascorbic acid (sample *A*) by subcutaneous injection of 5 mg./100 g. body weight twice daily; control courses with a nil intake were included for comparison; the products excreted in urine were measured. The effect of ACTH injections during a course of dehydroascorbic acid were also studied. The results for guinea pig *C* are summarized in Fig. 1. The other animal gave an identical response. When

Table 2. *Ascorbic acid of tissues after injection of ascorbic acid or dehydroascorbic acid (sample B) into guinea pigs*

Groups 1–3 were depleted for 12, 16 and 21 days respectively. Supplement was then administered for 3 days and animals killed on 4th day. Validity of ratios in brackets is doubtful because of low ascorbic acid concentration in the filtrates. Mean values are given.

				Adrenal gland			Liver		
Group no.	No. of animals	Supplement administered	Dose (mg./day)	Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material	Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material (mean ratio for the group)
1	3	None	None	4	6	—	0·4	1·1	—
	5	Ascorbic acid	10	37	39	0·97	5·8	9·2	0·72
	3	Dehydroascorbic acid	14	14	18	(0·70)	2·5	2·7	0·92
	5	Dehydroascorbic acid	28	34	39	0·94	5·5	6·6	0·84
2	4	Ascorbic acid	20	62	70	0·89	8·0	9·3	0·85
	4	Dehydroascorbic acid	40	59	69	0·87	8·4	8·9	0·94
3	3	None	None	1·5	1·0	—	1·0	0·4	—
	3	Ascorbic acid	10	50	50	1·03	6·6	8·2	0·80
	3	Dehydroascorbic acid	10	18	23	(0·72)	5·6	5·9	1·2

Table 3. *Effect of ACTH and cortisone on concentration of ascorbic acid and oxidation products in guinea pigs*

Guinea pigs were killed on the 16th day unless stated otherwise. Doses (days 13 to 15) injected subcutaneously were: ACTH, 20 i.u. of gel; ascorbic acid, 5 mg. and dehydroascorbic acid (sample *B*), 10 mg. twice daily; cortisone acetate 5 mg. once daily. Mean values are given.

				Adrenals			Liver			
Group no.	Daily treatment on days 1–12	Daily treatment on days 13, 14, 15	No. of animals	Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material*	Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material*	Total R & K material accumulated in liver (mg.)
1	Depleted	Killed on 13th day	3	4	6	—	0·4	1·1	—	—
2	Depleted	Depleted	3	5	3	—	0·8	0·4	—	—
3	Ascorbic acid, 20 mg.	Killed on 13th day	6	53	65	0·86	8·6	9·4	0·92	—
4	Cabbage, 100 g.	Killed on 13th day	6	54	65	0·86	6·3	7·6	0·81	—
5	Cabbage, 100 g.	Cabbage, 100 g.	3	67	75	0·92	17·9	22·2	0·81	—
6	Depleted	Ascorbic acid	3	37	39	0·97	5·8	9·2	0·72	1·45
7	Depleted	Ascorbic acid + ACTH	6	27	31	0·88	6·1	7·1	0·86	1·15
8	Depleted	Ascorbic acid + cortisone	5	44	45	1·00	5·0	6·3	0·83	0·97
9	Depleted	Dehydroascorbic acid	5	34	39	0·94	5·5	6·6	0·84	0·71
10	Depleted	Dehydroascorbic acid + ACTH	5	29	33	0·86	5·2	7·3	0·72	1·14
11	Depleted	Dehydroascorbic acid + cortisone	6	53	66	0·82	5·3	7·2	0·73	0·90

* Mean difference of ratios control – treated and t (degrees of freedom in parentheses): adrenals, group 7, +0·10, 1·4 (8); group 8, −0·02, 0·2 (9); group 10, +0·08, 1·0 (9) and group 11, +0·12, 1·0 (8); liver, group 7, −0·15, 1·5 (9); group 8, −0·11, 1·1 (10); group 10, +0·12, 1·5 (10) and group 11, +0·11, 1·3 (9).

Table 4. *Effect of ACTH on metabolism of natural and synthetic ascorbic acid and sodium ascorbate in guinea pigs*

Dose of ascorbic acid 200 mg. and of ACTH 60 i.u. soluble ACTH in 0·5 ml. saline. Groups 1–7 consisted of 4 and groups 8–16 of 5 guinea pigs each. Mean values are given.

		Time of killing after beginning expt. (hr.)	Adrenals			Liver		
Group no.	Injection		Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material*	Ascorbic acid (mg./100 g.)	R & K material (mg./100 g.)	Ascorbic acid/R & K material
1	None	0	109	109	1·01	14·0	12·8	1·10
2	None	2	101	99	1·02	—	—	—
3	ACTH	0·25	63·5	66	0·96	—	—	—
4	ACTH	0·5	86	90	0·96	—	—	—
5	ACTH	1	62	62	1·01	—	—	—
6	ACTH	2	84·5	81	1·06	22·0	19·6	1·12
7	ACTH	4	55	55	1·02	14·7	14·6	1·02
8	Ascorbic acid	0	63	60	1·03	—	—	—
9	Ascorbic acid	2	55·8	50·6	1·13	—	—	—
10	Ascorbic acid	6	63·4	67·0	0·95	—	—	—
11	Ascorbic acid + ACTH	2	56·2	56·4	1·00	—	—	—
12	Ascorbic acid + ACTH	6	39·0	39·0	1·00	—	—	—
13	Sodium ascorbate	2	75·8	78·4	1·00	—	—	—
14	Sodium ascorbate	6	78·0	80·0	1·05	—	—	—
15	Sodium ascorbate + ACTH	2	54	50·8	1·06	—	—	—
16	Sodium ascorbate + ACTH	6	48·6	48·6	1·00	—	—	—

* Mean difference of ratios control – treated and t (8 degrees of freedom): group 11, +0·13, 1·15; group 15, −0·06, 0·16; group 12, −0·05, 0·4 and group 16, +0·04, 0·3.

compared with the effects of injected ascorbic acid, injections of dehydroascorbic acid resulted in a considerable fall of ascorbic acid in urine and irregular changes in R & K material with a tendency for R & K material minus ascorbic acid to increase. The dehydroascorbic acid output in urine showed no significant change throughout and is therefore not shown. ACTH does not appear to influence the products excreted in urine during a course of dehydroascorbic acid.

In guinea pig *Z* subcutaneous injections of ascorbic acid (5 mg./100 g. body weight twice daily) were followed by subcutaneous injections of dehydroascorbic acid (14 mg./100 g. body weight twice daily; sample *B*). The output of ascorbic acid in urine averaged 4·1 and 2·7 mg./day respectively, while the R & K material averaged 4·4 and 6·1 mg./day.

The fate of dehydroascorbic acid in human subjects

Oral administration. After a course of ascorbic acid (600 mg./day in divided doses) sufficiently long to give a regular excretion of ascorbic acid, two normal human subjects (both male, aged 25) were given dehydroascorbic acid (sample *A*) for 4 and 5 days respectively. Then both were given ascorbic acid for a further control period. In subject 1 it was not realized at the time that the preparation of dehydroascorbic acid was only 80% pure, so that the effective dose was 480 mg./day; the ascorbic acid output (on dehydroascorbic acid) showed a fall consistent with this decrease in dosage. In subject 2 the effective dose was 600 mg./day, and there was no significant change in ascorbic acid output (Fig. 2). In both cases stopping the intake of ascorbic acid caused a sharp fall in urinary ascorbic acid. The excretion of ascorbic acid during the course of dehydroascorbic acid could not be due, therefore, to previously administered ascorbic acid.

Intramuscular administration. Comparison of the urinary output of ascorbic acid, dehydroascorbic acid and R & K material were made in subject 3 (female, aged 23, peptic ulcer) during periods of intramuscular injection of the acids. During a preliminary period of 9 days ascorbic acid was given orally until the output was regular. Ascorbic or dehydroascorbic acid was then injected four times daily. Plasma ascorbic and dehydroascorbic acid showed little change. During the course of dehydroascorbic acid the output of ascorbic acid fell to approximately one-third, and of R & K material to approximately one-half. There was no significant difference in the output of dehydroascorbic acid (Table 6).

To subject 4 (female, aged 39, rheumatoid arthritis) intramuscular injections of dehydro-

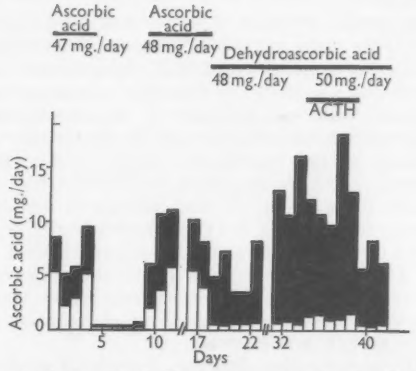

Fig. 1. Guinea pig *C*. Products excreted in urine during courses of subcutaneous injection of solutions of ascorbic acid and dehydroascorbic acid (sample *A*). The effect of 30 i.u./day of ACTH during the course of dehydroascorbic acid is also shown. Unshaded area represents ascorbic acid, total area R & K material.

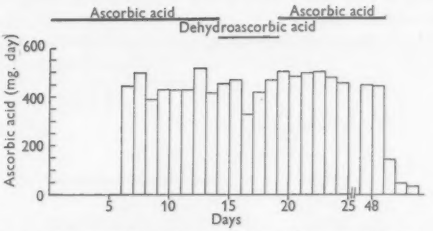

Fig. 2. Human subject 2. Ascorbic acid excreted in urine during courses of ascorbic acid and dehydroascorbic acid (sample *A*) taken by mouth. Doses, 600 mg./day.

Table 5. *Urinary excretion products of ascorbic acid and dehydroascorbic acid (sample B) from guinea pig G*

All doses were 500 mg. twice daily.

Days	Materia administered	Mean urine output (mg./day) Ascorbic acid	Dehydroascorbic acid	R & K material
1–7	Ascorbic acid, orally	0·4	0·5	1·3
8–16	Dehydroascorbic acid, orally	4·8	0·8	10·5
17	Dehydroascorbic acid, subcutaneously	84	0	256
18	Dehydroascorbic acid, subcutaneously (incomplete)	48	5	97

ascorbic acid were given after 6 days on a normal diet when she was moderately depleted of ascorbic acid. Results are given in Table 7. When dehydroascorbic acid was injected the urine ascorbic acid gradually rose to a stable value: R & K material, on the other hand, rose promptly. The plasma ascorbic acid also increased. On days 13 to 15 ACTH was also injected. The urinary output of ascorbic acid and R & K material showed little change, but the urinary dehydroascorbic acid was markedly increased.

In subject 5 (female, aged 39, rheumatoid arthritis) (Fig. 3) the effect of ACTH was studied during a course of dehydroascorbic acid. Ascorbic acid was given orally on days 1 to 6 in order to saturate the patient, and was followed by intramuscularly injected dehydroascorbic acid in four doses of 100 mg. daily. During the course of dehydroascorbic acid alone the output of metabolites became stable (days 18 to 22), the amounts in mg./day being: ascorbic acid, 105 ± 6; dehydroascorbic acid, $4{\cdot}2 \pm 1{\cdot}3$; R & K material, 276 ± 25. The effect of ACTH (days 23 to 27) was a brief rise in urinary ascorbic acid and R & K material; the plasma levels showed no significant change in ascorbic acid but an increase in R & K material.

Intravenous administration. A single intravenous injection of dehydroascorbic acid was given to subject 6 (male, aged 44, rheumatoid arthritis) who was receiving for therapeutic purposes 100 mg. of cortisone acetate daily, and the effect of dehydroascorbic acid was compared with that of ascorbic acid in the same dose given 3 days later (Fig. 4).

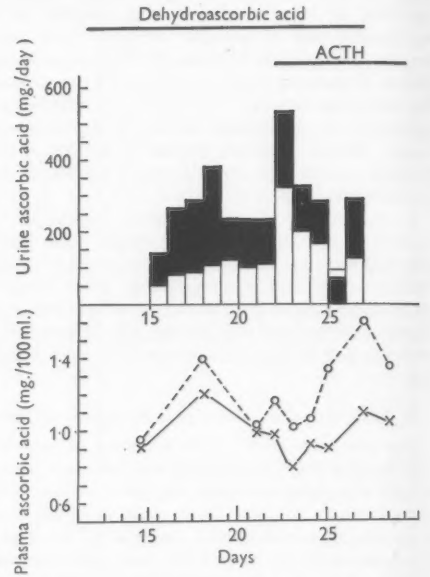

Fig. 3. Human subject 5. The effect of ACTH (50 i.u. at intervals of 6 hr.) on urine and plasma ascorbic acid during a course of intramuscular injections of dehydroascorbic acid (sample *B*, 400 mg./day). Symbols for plasma: O---O, R & K material; ×—×, ascorbic acid. Urine symbols as for Fig. 1.

Table 6. *Subject* 3. *Urinary excretion products after intramuscular injection of ascorbic acid and dehydroascorbic acid (sample B)*

All doses were 100 mg. four times daily.

Days	Material administered	Mean urinary output (mg./day) and S.E. Ascorbic acid	Dehydroascorbic acid	R & K material
10–13	Ascorbic acid	283 ± 12	$1{\cdot}5 \pm 0{\cdot}2$	366 ± 46
14–18	Dehydroascorbic acid	71 ± 11	$4{\cdot}2 \pm 1{\cdot}5$	186 ± 24
19–23	Ascorbic acid	214 ± 24	$6{\cdot}4 \pm 1{\cdot}5$	344 ± 32

Table 7. *Subject* 4. *Urinary excretion products after intramuscular injection of dehydroascorbic acid (sample B)*

On days 13 to 15, 60 i.u. of ACTH gel were also injected.

Days	Dose (mg./day)	Urinary output (mg./day) Ascorbic acid	Dehydroascorbic acid	R & K material	Plasma ascorbic acid (mg./100 ml.) Ascorbic acid	Dehydroascorbic acid
1–6	—	8·5	0	0	0·20	0·09
7	830	14	2	103	0·35	0·24
8	830	12	2	115	—	—
9	830	40	4	150	—	—
10–12	400	126 ± 15	$5{\cdot}7 \pm 0{\cdot}7$	162 ± 16	1·30	0·14
13–15	400	129 ± 11	$14 \pm 2{\cdot}6$	196 ± 17	0·97	0·33

After dehydroascorbic acid the plasma ascorbic acid and R & K material increased, but the rise was smaller than after ascorbic acid; the difference between plasma ascorbic acid and R & K material increased slightly on both occasions. Plasma dehydroascorbic acid, after both injections, showed only small irregular changes.

Effect of ACTH on metabolism of dehydroascorbic acid given intravenously

In subject 7 (female, aged 34, lupus erythematosus) advantage was taken of the intravenous route in an attempt to demonstrate whether ACTH injections might result in a persistence of dehydroascorbic acid; such a persistence should result in an increase in plasma or urinary dehydroascorbic acid.

In order to raise the plasma and urine concentration of ascorbic acid and R & K material to levels at which they could be determined with accuracy, ascorbic acid was given daily in 50 mg. oral doses at 5, 7 and 10 p.m. throughout the investigation. After 11 days for equilibration, a single intravenous injection of 180 mg. of dehydroascorbic acid was given at 10 a.m. on day 12. After 4 days, to permit changes in ascorbic acid saturation to subside, dehydroascorbic acid was injected at 11 a.m., 1 hr. after intramuscular injection of 100 i.u. ACTH in saline; for therapeutic purposes 50 i.u. of ACTH in

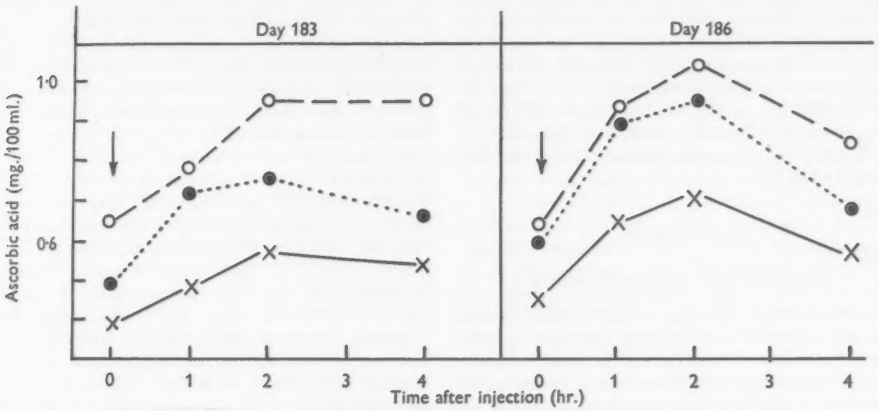

Fig. 4. Human subject 6. Plasma changes following intravenous injections of ascorbic acid and dehydroascorbic acid (sample *B*). This subject was receiving 100 mg. of cortisone acetate daily (since day 92). Dehydroascorbic acid (200 mg.) was given at time indicated by arrow on day 183 and ascorbic acid (200 mg.) on day 186. O- - -O, R & K material; ●....●, dehydroascorbic acid plus ascorbic acid; ×—×, ascorbic acid.

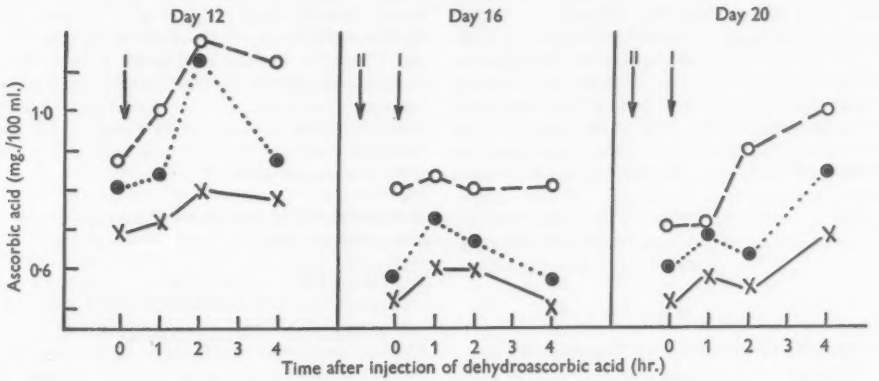

Fig. 5. Human subject 7. The effect of ACTH on the plasma ascorbic acid, dehydroascorbic acid and R & K material. Symbols as in Fig. 4. Arrows indicate: I, injection of dehydroascorbic acid (sample *B*); II, injection of ACTH. For exact dosage see text.

saline were given at 5 p.m., 10 p.m., 4 a.m. and 10 a.m. daily through the remainder of this investigation. A third injection of dehydroascorbic acid was given on day 20 at 11 a.m., 1 hr. after 50 i.u. of ACTH. Plasma concentration of ascorbic acid, dehydroascorbic acid and R & K material were determined immediately before and 1, 2 and 4 hr. after each injection of dehydroascorbic acid (Fig. 5); the ascorbic acid level increased to approximately the same degree on all three occasions, indicating some reduction of the intravenous dehydroascorbic acid. ACTH did not significantly affect the amount of dehydroascorbic acid or the ratio of ascorbic acid/R & K material. Excretion of ascorbic acid, dehydroascorbic acid and R & K material is shown in Table 8. The first injection of dehydroascorbic acid, on day 12, was followed by an increased excretion of 32 mg. of ascorbic acid and 101 mg. of R & K material as compared with the average for days 10 and 11. Comparison of days 16 and 12 indicates a significantly increased excretion of R & K material minus ascorbic acid after the first injection of ACTH.

DISCUSSION

Metabolism of dehydroascorbic acid

The antiscorbutic effect of dehydroascrobic acid when administered to guinea pigs either orally or by injection confirms the findings of earlier workers using oxidized ascorbic acid solutions. By injection, as little as 2 mg. daily was sufficient to prevent the onset of scurvy and to permit continued growth. By feeding, this was insufficient to allow continued gain in weight. That dehydroascorbic acid is readily reduced after injection into the guinea pig is also demonstrated by its uptake as ascorbic acid in the tissues. Thus 40 mg. daily for 3 days were sufficient to raise the heavily depleted adrenal and liver ascorbic acid to nearly normal levels, although the experimental conditions were deliberately chosen to avoid the supersaturation period after giving a dose (Penney & Zilva, 1946). The injection of 10 mg. dehydroascorbic acid failed to raise the tissue level to normal. This dosage was also too small to cause a significant excretion of ascorbic acid or R & K material in urine and, since the whole normal animal would not contain more than 8 mg. of ascorbic acid, an appreciable portion must have been destroyed. It did not persist as dehydroascorbic acid in the tissues and it was not present as an oxidation product recognizable by the Roe & Kuether procedure. Damron *et al.* (1952), using a different method, concluded that at most only trace amounts of dehydroascorbic acid were present in normal tissues; Penney & Zilva (1943) found none. Oxidation may proceed further to oxalate and carbon dioxide (Burns, Burch & King, 1951).

In the guinea pig, after injection of dehydroascorbic acid, only small amounts of excretory products were detected and dehydroascorbic acid was not present in the urine in detectable amounts. When equal doses of ascorbic and dehydroascorbic acids were compared, the latter led to the excretion of much less ascorbic acid and much more R & K material minus ascorbic acid. This tendency was also observed in the human subjects. When dehydroascorbic acid was injected into an individual unsaturated with ascorbic acid, the excretion of R & K material minus ascorbic acid was high for the first few days, but it was several days before the ascorbic acid in urine rose sharply. This also suggests that the oxidation products of dehydroascorbic acid are taken up by the unsaturated organism to a much smaller degree (if at all) than the ascorbic acid formed by reduction in the body. This finding was also confirmed after intravenous administration of dehydroascorbic acid to the more nearly saturated subject 7.

After injection of dehydroascorbic acid intramuscularly or intravenously there is a temporary rise in the plasma ascorbic acid level and a tendency to an even greater increase in the R & K material minus ascorbic acid (Figs. 4 and 5). Plasma dehydroascorbic acid, on the other hand, followed the same trend as the ascorbic acid. It must be concluded that injected dehydroascorbic acid is partly reduced to ascorbic acid but that a greater proportion is oxidized to products estimated by the Roe & Kuether method. It does not tend to persist as dehydroascorbic acid.

Table 8. *Subject* 7. *Urinary excretion products following intravenous injection of dehydroascorbic acid with and without ACTH*

For details of administration see text.

Day	Injection	Urinary output (mg./day)		
		Ascorbic acid	Dehydroascorbic acid	R & K material
10	None	94	6	200
11	None	99	5	189
12	Dehydroascorbic acid	129	1	296
16	ACTH + dehydroascorbic acid	128	4	347
20	ACTH + dehydroascorbic acid	119	0	268

Comparison of results with orally administered dehydroascorbic acid with those from injection experiments shows interesting differences. In the guinea pig dehydroascorbic acid given orally is partially reduced to ascorbic acid as evidenced by its antiscorbutic properties, although large amounts lead to the excretion of only minimal amounts of ascorbic acid or R & K material. This is in agreement with the results of Penney & Zilva (1943, 1946), who demonstrated reduction of dehydroascorbic acid in the stomach wall; absorption of ascorbic acid in the stomach was poor and destruction of it took place in the more alkaline small intestine. In the human, on the other hand, absorption is better and appears as good as that of ascorbic acid (Fig. 2). The fact that the same dose of ascorbic and dehydroascorbic acid given orally in the human leads to excretion of similar amounts of ascorbic acid in the urine suggests that the latter is almost quantitatively reduced to ascorbic acid. This is in accord with the results of Johnson & Zilva (1934), Todhunter *et al.* (1950) and de Ritter *et al.* (1951). The reduction of dehydroascorbic acid by the oral route is somewhat better than after injection, when a smaller amount of ascorbic acid is excreted in urine after a dose of dehydroascorbic acid than after an equivalent dose of ascorbic acid. It is possible that bacterial reduction may play some part in the intestinal conversion of dehydroascorbic acid into ascorbic acid (Mapson & Ingram, 1951).

In the experiments in which tissue levels of ascorbic acid were studied in guinea pigs after injection of dehydroascorbic acid 2 to 3 times as much dehydroascorbic acid was required to raise the tissue concentration of ascorbic acid to the level produced by a given amount of ascorbic acid. When the antiscorbutic activity is considered, 2 mg. daily of dehydroascorbic acid is less effective by injection than 2 mg. of ascorbic acid. In both the guinea pig and man approximately 3 times as much dehydroascorbic acid as ascorbic acid had to be injected to produce a similar excretion of ascorbic acid (guinea pig *Z* and Table 6).

Caution should be adopted with the injection of dehydroascorbic acid into human subjects. Apart from the complaints by the patients of local pain, the liver and epidermal changes in the guinea pig are of special note. These appeared to be caused by dehydroascorbic acid or by some impurity only present in very small amounts, since they were observed with both samples of dehydroascorbic acid. It is possible that the toxic effects of dehydroascorbic acid in the guinea pig may be due to decreased ability of this animal to reduce the acid, even though the reduction is sufficient to give tissue levels of ascorbic acid incompatible with the development of scurvy. The ultimate cause of the toxicity is not at present known.

Effects of ACTH and cortisone on dehydroascorbic acid metabolism

In guinea pigs we failed to observe any change in ascorbic or dehydroascorbic acid metabolism when ACTH or cortisone acetate were given. This was even true when conditions of possible utilization of ascorbic acid in the adrenal were chosen, i.e. during the fall occurring in the few hours immediately after ACTH injection (Long, 1947). Neither in this short-term experiment nor the longer one lasting over 3 days was there a significant fall in liver ascorbic acid concentration such as that observed by Harris, Bland, Hughes & Constable (1953). It is possible that even in the longer experiment the duration of ACTH or cortisone administration was too short.

The rise in excretion of ascorbic acid and R & K material when ACTH and ascorbic acid was administered, observed by McSwiney *et al.* (1954), was probably not seen because the period of observation was too short. In the human subjects ACTH produced more definite changes. In the excretion pattern in subject 5 there was a transitory increase in ascorbic acid and R & K material minus ascorbic acid, but the ratio of ascorbic acid/R & K remained virtually unaltered, as it did in the experiments employing ascorbic acid (McSwiney *et al.* 1954). However, the sharp rise in urinary R & K material minus ascorbic acid observed when intravenous dehydroascorbic acid and ACTH were given together must be noted (Table 8). Another subject (Table 7) excreted small but significantly increased amounts of dehydroascorbic acid in the urine when this acid and ACTH were given. ACTH caused a similar increase in dehydroascorbic acid in one subject receiving ascorbic acid (McSwiney *et al.* 1954). In two instances there was a fall in the plasma ascorbic acid/R & K ratio (Table 7 and Fig. 3). This effect, too, may be observed in the experiments of McSwiney *et al.* (1954). We have never seen a plasma ascorbic acid/R & K ratio approximating to 1·0 as reported by Stewart, Horn & Robson (1953). These authors found the change occurred within 4 hours of the injection of ACTH or oral administration of cortisone acetate, whereas our observations were made over a period of several days. It may be concluded that ACTH does not apparently regularly and significantly retard the reduction of dehydroascorbic to ascorbic acid and this is especially true in the guinea pig experiments.

SUMMARY

1. The metabolism of dehydroascorbic acid has been studied. Injected into guinea pigs this substance had an antiscorbutic effect and raised the tissue level of ascorbic acid. Dehydroascorbic acid did not appear in the tissues or urine. In urine

there were increased amounts of oxidation products estimated by the Roe & Kuether method. Much of the injected material was not accounted for.

2. In guinea pigs, injected dehydroascorbic acid produced toxic symptoms which include atrophic changes in the fur, fatty liver and death.

3. In human subjects oral dehydroascorbic acid behaved quantitatively like ascorbic acid.

4. Dehydroascorbic acid methanolate was safely injected into human subjects. It was largely reduced to ascorbic acid. The plasma level and urinary excretion of ascorbic acid increased. Approximately 3 mg. of dehydroascorbic acid were equivalent to 1 mg. of ascorbic acid in this respect.

5. Dehydroascorbic acid injected into human subjects led in plasma and urine to a greater rise in oxidation products than in ascorbic acid. Dehydroascorbic acid did not appear in the urine.

6. Cortisone and ACTH did not modify the tissue metabolism of dehydroascorbic acid injected into guinea pigs. In particular, oxidation products of ascorbic acid did not appear in the adrenals.

7. In one human subject the amount of ascorbic acid in the urine increased with ACTH. In one subject the oxidation products increased and in another there was a small rise in dehydroascorbic acid excretion.

Our thanks are due to Dr W. A. R. Thompson who arranged a supply of dehydroascorbic acid by Messrs Boots Ltd.; to Dr A. L. Morrison of Roche Products Ltd. for supply of dehydroascorbic acid methanolate. ACTH and cortisone were supplied by the Medical Research Council. We are indebted to Dr J. M. Tanner for considerable help with the statistical analyses; to Mr A. L. Wooding for photographic assistance.

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β-Glucosidase from Rumen Liquor

PREPARATION, ASSAY AND KINETICS OF ACTION

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(*Received* 10 *March* 1954)

The most important feature of ruminant digestion is the utilization of cellulose and other fibrous plant substances. There is as yet no clear evidence of the steps occurring during the initial breakdown of these materials, but it appears probable that some form of enzymic depolymerization takes place, followed by further splitting of glycosidic linkages. This would finally result in the production of simple sugars, which could easily be transformed into the volatile fatty acids known to be the main end-products of polysaccharide digestion that are absorbed from the rumen. It seems likely that, in the case of cellulose at least, β-glucosidase plays an important part in such a scheme, and it was with this in mind that a study of this enzyme in the rumen of the sheep was undertaken.

For preliminary studies on rumen β-glucosidase, it was decided to use a chromogenic substrate, to enable the rumen liquor to be treated as one whole tissue, and thus ensure that no important active fractions are overlooked. Such a procedure was adopted successfully by Karunairatnam & Levvy (1951) in their investigation of a glucuronide-decomposing enzyme in sheep rumen. After synthesizing and testing several chromogenic β-D-glucosides, *o*-nitrophenyl β-D-glucoside was finally

selected for the work described in this paper. *p*-Nitrophenyl β-D-glucoside, which was first used by Aizawa (1939), has been employed extensively in β-glucosidase studies, mainly by Japanese workers, and *o*-nitrophenyl β-D-galactoside has been used by Lederberg (1950), who investigated β-galactosidase from *Escherichia coli*.

EXPERIMENTAL

Materials. *o*-Nitrophenyl β-D-glucoside was prepared using the method described by Seidman & Link (1950) for *o*-nitrophenyl β-D-galactoside. The product, recrystallized from ethanol, had m.p. 152° and $[\alpha]_D^{16} - 106{\cdot}6°$ in water (*c*, 1·2), properties identical with those of the compound described by Montgomery, Richtmyer & Hudson (1942).

Sodium gluconate and glucono-1:5-lactone were both obtained from British Drug Houses Ltd.; the glucono-1:5-lactone was twice recrystallized from ethanol before use: m.p. 153°; $[\alpha]_D^{18} + 63{\cdot}1°$ in water (*c*, 1·8). Glucono-1:4-lactone was prepared from calcium gluconate as described by Hedenburg (1915), and recrystallized from ethanol: m.p. 135°; $[\alpha]_D^{18} + 67{\cdot}0$ in water (*c*, 1·8). Rumen liquor, obtained as described below, was strained through nine layers of surgical gauze before use.

Enzyme assay. As a result of preliminary experiments, the following procedure was adopted. To 3 ml. of 0·2M-Na_2HPO_4–0·1M citric acid buffer (McIlvaine, 1921), pH 5·4, was added 0·5 ml. of 0·04M *o*-nitrophenyl β-D-glucoside solution (giving a final concentration in the incubation mixture of 5 mM) and 0·5 ml. of the enzyme preparation. The mixture was incubated for 1 hr. at 37°, and the reaction then stopped by the addition of 2 ml. 5% (w/v) trichloroacetic acid solution. After centrifuging at 1500*g* for 15 min., 4 ml. of the supernatant were measured into another tube, and 1·5 ml. of 0·5N-NaOH added, followed by 2·5 ml. of 0·25M glycine–Na_2CO_3 buffer, pH 10·0. The intensity of the yellow-orange colour developed was measured in a Spekker photoelectric absorptiometer (Hilger & Watts Ltd., London, N.W. 1), using Ilford no. 601 (425 mμ.) violet filters, and the weight of liberated *o*-nitrophenol was measured from a calibration curve. Blanks were carried out for enzyme and substrate incubated separately.

Inhibition experiments. For inhibition experiments, the following modification of the above procedure was adopted. To 2·5 ml. of 0·2M-Na_2HPO_4–0·1M citric acid buffer, pH 5·4, were added 0·5 ml. of 0·04M *o*-nitrophenyl β-D-glucoside, 0·5 ml. inhibitor solution (or 0·5 ml. of water in the case of the control) and 0·5 ml. enzyme preparation. After incubation the normal assay procedure was followed. Where necessary the pH of the inhibitor solution was adjusted to 5·4 at the glass electrode before use, but this was unnecessary in the case of the lactone solutions used.

Sampling regime and diet. The animals used were Cheviot sheep fitted with permanent rumen fistulae. Their daily diet consisted of 500 g. hay at 7 a.m. and a similar quantity at 4 p.m., with free access to water and salt lick. Rumen liquor was always withdrawn at 9.30 a.m., not more than 1500 ml. being removed from each sheep at any time.

Preparation of cell-free extracts of the enzyme. The required fraction of the rumen contents was obtained by centrifuging (see below), washed 4 times on the centrifuge with NaCl (1%, w/v; 10 ml./ml. of deposit) and suspended in about 5 times its own volume of water. Washing with 1% NaCl removed no enzyme activity. The suspension was then poured, with stirring, into 4 times its volume of acetone, stirring being continued at room temperature for 40 min. After centrifuging at 1500*g* for 6 min., the deposit was well washed with half the previous volume of acetone, and centrifuged for a further 6 min., after which it was dried overnight *in vacuo* at room temperature. The dried powder was suspended in an 0·05% (w/v) solution of the non-ionic surface-active agent Triton X-100 (Rohm and Haas Co., Philadelphia, U.S.A.) using 60 ml./g. acetone powder and, after grinding in a glass homogenizer, was extracted at room temperature, with stirring, for 4 hr. After centrifuging at 10000*g* for 30 min. in an M.S.E. High Speed Angle-13 Centrifuge, a second extraction of the debris was carried out using half the previous volume of Triton. The supernatants in each case were recentrifuged and then combined. Fifty per cent of the total activity was extracted. It was found that Triton X-100 gave a better extraction (about 20% more) than either water alone, or buffers of varying composition and pH. Control experiments showed that the above concentration of Triton had no effect on the activity of an aqueous extract of acetone-dried powder, and did not interfere with the colour reaction for *o*-nitrophenol determination. Except where stated otherwise, all experiments described in this paper were carried out using enzyme solution prepared by this method from combined Fractions I and II (see below).

Nitrogen estimations. Digestion was carried out according to the method of Chibnall, Rees & Williams (1943). The ammonia was removed by steam distillation using the apparatus of Markham (Markham, 1942), absorbed in 2% boric acid containing methyl red and bromocresol green (Conway, 1947) and titrated with 0·01N-HCl.

RESULTS

Fractionation of rumen liquor and estimation of enzyme activity in the whole-cell fractions. To ascertain in which size of particle the main β-glucosidase activity lay, fractionation of the rumen liquor was carried out by centrifuging at various speeds for predetermined periods, and washing and assaying the fractions so obtained (Table 1). Centrifuging in this manner gave a rough separation of the rumen liquor micro-organisms into protozoa (fraction I), the larger bacteria with some small protozoa (fraction II), and the smaller bacteria (fraction III). There were probably also plant fragments in all fractions (see below). The optically clear supernatant from fraction III had no activity, indicating that there was no β-glucosidase present in solution in rumen liquor. By far the greatest total activity lay in fraction II. Since further experiments, described below, showed fractions I and II to give enzyme preparations of similar properties, these fractions were in later experiments combined and used in the preparation of extracts of β-glucosidase.

Variation in activity with pH. The variation in relative activity with pH of an extract of an acetone-dried powder from fractions I and II combined is

Table 1. *Distribution of β-glucosidase activity in fractions obtained by centrifuging rumen liquor*

Strained rumen liquor (100 ml.) was separated, by centrifuging, into the fractions detailed below, each fraction being washed four times with NaCl (100 ml.; 1%, w/v) and finally suspended in water. Samples of each fraction (0·5 ml.) were assayed using the standard conditions described in the text. Fractions I and II were obtained using M.S.E. centrifuges with swing-out heads, while fraction III was obtained using an M.S.E. High Speed Angle-13 Centrifuge.

				Enzyme activity	
Fraction	Relative centrifugal force (*g*)	Period of centrifuging (min.)	Final vol. of fraction (ml.)	μg. *o*-nitrophenol/ mg. N	Total *o*-nitrophenol liberated (μg.)
I	15	2	20	2032	5 880
II	1 500	30	100	1676	31 800
III	10 000	30	70	1323	5 040
IV	Supernatant (cell-free)				0
Strained rumen liquor (uncentrifuged)			100	608	41 100

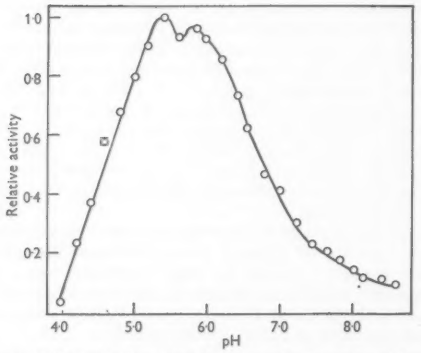


Fig. 1. Effect of pH on the liberation of *o*-nitrophenol from 5 mM *o*-nitrophenyl β-D-glucoside. A washed suspension of fractions I and II combined (see text) was used in 0·2 M-Na_2HPO_4–0·1 M citric acid buffer of appropriate pH, and the normal assay procedure described in the text carried out. The final assay sample was brought to pH 10·0 with 0·5 N-NaOH, and adjusted to a final volume of 8 ml. with 0·25 M glycine–Na_2CO_3 buffer pH 10·0. An activity of 1·0 is equivalent to 309 μg. *o*-nitrophenol liberated/hr.

shown in Fig. 1. The pH of the buffers was varied by increments of 0·2 unit within the range 3·6–8·4, the pH of each hydrolysis mixture being measured before and after incubation, and the average value taken. It was found that the pH altered only slightly during incubation. Determinations of the variation in activity with pH were also carried out on the original suspensions of all the fractions separated as described above, as well as on extracts of acetone-dried powders of each of these separate fractions. In every case the same pattern of curve was obtained, as shown in Fig. 1, with peaks of about the same relative activity at pH 5·4 and 5·8.

Most of the later experiments described were carried out at pH 5·4, though in certain cases (mentioned below) a duplicate experiment was performed at pH 5·8.

The activity of an extract of an acetone-treated powder of combined fractions I and II in H_3PO_4–NaOH, phthalic acid–KOH, citric acid–NaOH, and sodium acetate–acetic acid buffers of 0·15 M concentration at pH 5·4 was the same as that in 0·2 M-Na_2HPO_4–0·1 M citric acid buffer. A determination of the pH optimum in sodium acetate–acetic acid buffer gave the same pattern of curve as shown in Fig. 1 for Na_2HPO_4–citric acid buffer.

Variation in activity with substrate concentration Fig. 2*a* shows the effect on the activity of the enzyme at pH 5·4 of varying the substrate concentration. The maximum activity of the preparation was reached at a substrate concentration of 5 mM. In no experiment was inhibition by excess substrate noted. The results were analysed by the graphical method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. The results in Fig. 2*a* gave a value of 0·88 mM for K_m, the dissociation constant of the enzyme–substrate complex (Fig. 2*b*). A mean value of 0·86 mM was obtained for K_m in six experiments. A determination at pH 5·8 gave a value of 0·80 mM. A substrate concentration of 5 mM was selected for routine assay purposes.

Effect of varying the duration of hydrolysis. The rate of hydrolysis of *o*-nitrophenyl β-D-glucoside is linear with time for 3–4 hr. (Fig. 3), thus showing that the preparation is stable at 37° and saturated with substrate for that period. Where necessary, the pigment was diluted with glycine buffer before measuring its colour intensity.

Effect of varying the enzyme concentration. The effect on the activity of varying the enzyme concentration over a tenfold range is shown in Fig. 4. Since the reaction velocity is proportional to the concentration, it would appear that there is no natural inhibitor or substrate present in the preparation. The presence of either would have caused a change in the slope of the activity/concentration curve, since their concentration would have varied as the enzyme concentration was altered (see Levvy & Marsh, 1954). If necessary, the pigment was diluted as described in the preceding section.

Variation in stability with pH. The variation in stability of the enzyme with pH is shown in Fig. 5. Similar curves were obtained with incubation periods of 15 min., 1 hr. and 2 hr., indicating that the variation in stability was an instantaneous effect of extremes of pH, rather than of incubation at these pH values. The enzyme was quite stable at the pH of optimum activity.

It is also clear from a comparison with the pH optimum curve (Fig. 1), that the shape of the latter is, in the lower range of pH, dependent on the fact that there is considerable inactivation there.

β-Glucosidase in the hay diet. Davidson (1954), investigating plant pigments in the rumen of sheep, found that all microbial fractions obtained by centrifuging were to a greater or lesser extent contaminated by pigmented plant particles from the diet. In view of this, the hay fed to the sheep was examined for β-glucosidase activity. A sample of the hay was ground in a hammer mill to pass a sieve of mesh 0·55 mm. The ground hay (1 g.) was suspended in water (40 ml.) and incubated for 4 hr. at 37°, after which an assay was carried out. This suspension was then put through the treatment described for rumen liquor, assays being performed

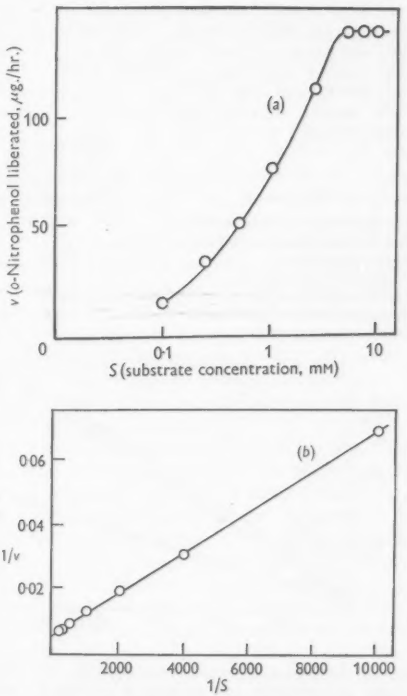


Fig. 2. Effect of variation in the concentration of *o*-nitrophenyl β-D-glucoside on the liberation of *o*-nitrophenol by rumen β-glucosidase (a cell-free extract prepared as described in text) in 0·2M-Na_2HPO_4–0·1M citric acid buffer, pH 5·4, after incubation for 1 hr. at 37°. (*a*) Plotting the substrate concentration (*S*) against the rate of decomposition (*v*). (*b*) Plotting 1/*S* against 1/*v* (Lineweaver & Burk, 1934).

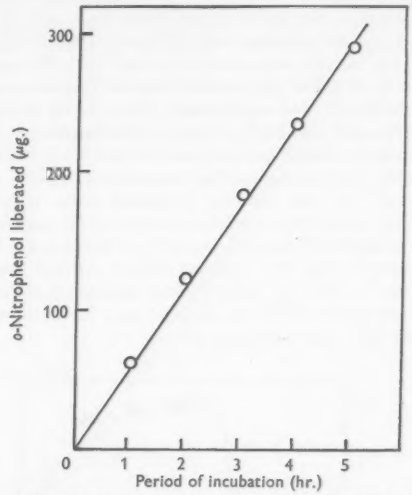


Fig. 3. Effect of period of incubation at 37° on the hydrolysis of 5 mM *o*-nitrophenyl β-D-glucoside by a cell-free extract of the enzyme in 0·2M-Na_2HPO_4–0·1M citric acid, pH 5·4. Other conditions as for standard assay procedure (see text).

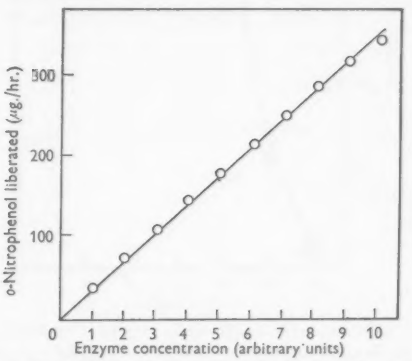


Fig. 4. Relationship between decomposition of *o*-nitrophenyl β-D-glucoside (5 mM) and concentration of the enzyme over a tenfold range. Standard conditions of assay as described in text. The activity value at concentration '10' is that of a cell-free extract obtained from an acetone-dried powder of fractions I and II combined; 0·5 ml. of this extract liberated 350 μg. *o*-nitrophenol/hr. The other concentrations were obtained by dilution of this extract, 0·5 ml. of the diluted solution being used for each assay.

at various intermediate stages (see Table 2). On washing the suspension of hay 4 times with 1% NaCl (80 ml. each time), suspending the washed deposit in 50 ml. of water, and carrying out an assay, it was found that 63% of the total activity in the original suspension had been removed. An acetone-treated powder of the suspension was prepared as for rumen liquor fractions, and the dried powder extracted with Triton X-100 (0·05%; 30 ml.). Assays were carried out on both the suspension of the whole acetone-treated powder, and on the centrifuged supernatant. The activity in this supernatant was 9·5% of that in the suspension of the whole acetone-treated powder, and 3·9% of the activity in the original hay suspension. This is in contrast to the enzyme prepared from rumen liquor, which did not pass into solution on washing the centrifugal fractions, and 50% of which could be extracted from the acetone-treated powder with Triton X-100. It would thus appear that the enzyme dealt with in the rumen liquor fractions was essentially microbial in origin.

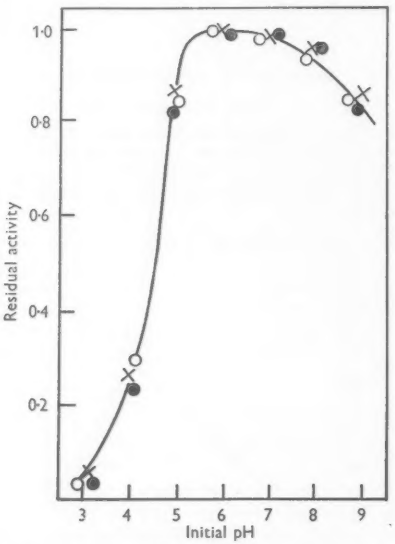


Fig. 5. Effect on the activity of the enzyme of incubation at varying pH at 37° for 15 min. (○—○), 1 hr. (×—×), and 2 hr. (●—●). A cell-free extract (4 ml.), was adjusted with HCl or NaOH to the required pH and incubated at 37° for the above periods of time, after which the solution was adjusted to pH 5·4, the volume made up to 10 ml., and the standard assay procedure carried out (see text) using 0·5 ml. of the solution. The results are expressed as fractions of the activity of a similarly diluted solution of a cell-free extract which was not incubated. An activity of 1·0 corresponds to 292 μg. *o*-nitrophenol liberated/hr.

Inhibition by gluconolactones

Both glucono-1:4-lactone and glucono-1:5-lactone were found to be strong inhibitors of rumen β-glucosidase activity. Measurements of enzyme activity were carried out in the presence and absence of 0·1 mM solutions of glucono-1:4-lactone and glucono-1:5-lactone, using concentrations of substrate varying from 0·1 to 7·5 mM. The results were analysed by the graphical method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$ (Fig. 6). In both cases, the lines obtained in presence and absence of inhibitor had a common intercept $1/V$ (V = maximum velocity), indicating that inhibition was competitive. Determination of K_i, the dissociation constant of the enzyme–inhibitor

Table 2. *Measurement of β-glucosidase activity in hay*

Suspension of milled hay (1 g.) in water (40 ml.) incubated for 4 hr. at 37°, and then centrifuged and sediment washed with 1% NaCl (4 × 80 ml.). Resuspended in water (50 ml.) and precipitated with acetone (200 ml.). Acetone-treated powder suspended in Triton X-100 solution (30 ml.; 0·05%) and solid centrifuged off. Assays carried out on various fractions under standard conditions described in text.

Treatment	Total activity in 1 g. hay (μg. *o*-nitrophenol liberated/hr.)
Whole hay suspension in water (after 4 hr. incubation at 37°)	6160
Washed suspension (resuspended in water)	2300
Acetone-treated powder in suspension	2520
Supernatant from suspension of acetone-treated powder	240

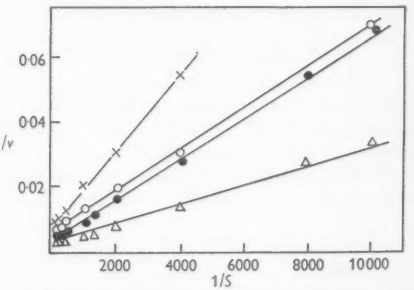


Fig. 6. Relationship between concentration of *o*-nitrophenyl β-D-glucoside and activity of the enzyme (*a*) in presence (●—●) and in absence (△—△) of 0·1 mM glucono-1:4-lactone; (*b*) in presence (×—×) and in absence (○—○) of 0·1 mM glucono-1:5-lactone, analysed by the method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. Different enzyme preparations (cell-free extracts) were used in *a* and *b*. The standard conditions for inhibition experiments (see text) were used. For units of v and S see Fig. 2.

complex gave, in the experiments shown, values of 0·08 mM for the 1:4-lactone, and 0·072 mM for the 1:5-lactone.

Mean values obtained for K_i were 0·094 mM for glucono-1:4-lactone from three experiments and 0·091 mM for glucono-1:5-lactone from three experiments. Similar values were obtained with 0·2 mM solutions of the lactones. The lactones thus each had an affinity for the enzyme about ten times that of *o*-nitrophenyl β-D-glucoside. Gluconic acid, in the form of sodium gluconate, showed only very slight inhibitory power, about 4·9% inhibition at millimolar concentration. Of the other compounds tested as possible inhibitors at millimolar concentration, saccharate, boiled and unboiled (see Levvy, 1952), showed no inhibitory power, while thiophenyl β-glucoside caused 5·4% inhibition.

Variation in inhibition with concentration of lactones. The effect on the activity of the preparation of varying the concentration of the lactones is shown in Fig. 7 which gives curves for glucono-1:4-lactone and glucono-1:5-lactone over the range 0·01 to 10 mM. Fifty per cent inhibition was obtained with concentrations of 0·25 mM glucono-1:4-lactone and 0·20 mM glucono-1:5-lactone. Similar curves were obtained when the experiments were repeated in 0·2M-Na_2HPO_4–0·1M citric acid buffer, pH 5·8.

Effect of time and temperature on the inhibitory power of aqueous solutions of lactones. Both glucono-1:4-lactone and glucono-1:5-lactone mutarotate when in aqueous solution, though at different rates (Hedenburg, 1915; Nef, 1914), and it was observed that the changes in rotation were accompanied by corresponding changes in inhibitory power. Fig. 8 shows the results of a series of such experiments, carried out at room temperature (19°). The solutions used for observing the changes in rotation of the lactones were, after suitable dilution, also used for the inhibition experiments.

It should be noted that although final equilibrium has not been reached in either case, the 1:5-lactone is closely approaching it, while the 1:4-lactone would require a much longer period (about 14 days, according to Hedenburg). Nevertheless, the fall in rotation, due to formation of gluconic acid, which has $[\alpha]_D - 6·7°$ (Rehorst, 1928), is followed in every case by a comparable drop in inhibitory power. The amount of lactone present at each time interval can be calculated from the percentage inhibition observed (using Fig. 7); the amounts so obtained agree well with those calculated from the observed rotations, assuming that gluconic acid is being produced, and that it has no inhibitory power. The percentage of glucono-1:4-lactone remaining after 119 hr. at room temperature was 59% (calculated from the percentage inhibition data of Fig. 7), and 63% (calculated from the rotation measurements). In the case of the glucono-1:5-lactone solution the values obtained are 18% from inhibition, and 19% from rotation measurements.

On heating a solution of either lactone to 100°, equilibrium is fairly rapidly reached, with a preponderance of 1:4-lactone and gluconic acid. Table 3 shows the changes in inhibition and rotation of solutions of both lactones over a period of 24 hr. at 100°. Again, the amounts of lactone calculated from the percentage inhibition using Fig. 7 (assuming

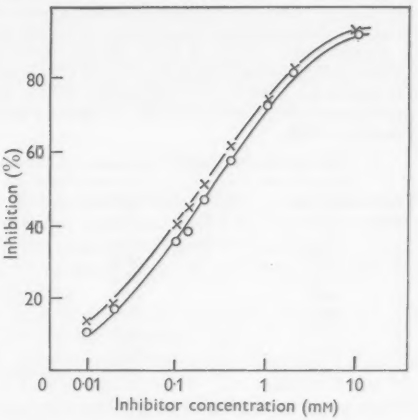


Fig. 7. Relationship between the percentage inhibition of the decomposition of 5 mM *o*-nitrophenyl β-D-glucoside and the concentration of glucono-1:4-lactone (○—○) and glucono-1:5-lactone (×—×). The enzyme preparation was a cell-free extract, and the standard conditions for inhibition experiments were used (see text).

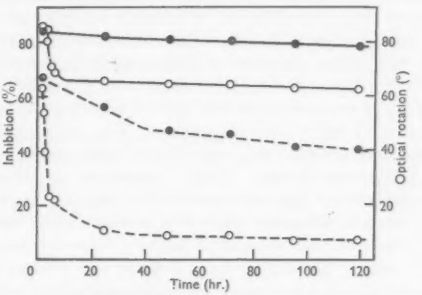


Fig. 8. Changes with time in inhibitory power of 2·5 mM glucono-1:4-lactone (●—●) and 2·5 mM glucono-1:5-lactone (○—○) and rotation of glucono-1:4-lactone (●---●) (*c*, 1·8 in water) and glucono-1:5-lactone (○---○) (*c*, 1·8 in water) at room temperature (19°). The enzyme preparation was a cell-free extract prepared as described in the text. In the inhibition experiments the solutions used in measuring optical rotation of the lactones were diluted to give a final incubation concentration of 2·5 mM. The standard conditions for inhibition experiments (see text) were used.

Table 3. *Changes with time in inhibitory power and rotation of aqueous solutions of glucono-*1:4*-lactone and glucono-*1:5*-lactone at* 100°

For inhibitory power tests the solutions used in measuring optical rotation were finally diluted to millimolar concentration and the standard procedure for inhibition experiments (see text) was used. The enzyme preparation was a cell-free extract of Fractions I and II combined. Calculations from the inhibition determinations (using Fig. 7) of the percentage of lactone remaining after 24 hr. at 100°, give values of 66% for both the glucono-1:4-lactone and glucono-1:5-lactone solutions. From the rotation measurements values of 65% for the 1:4-lactone solution and 61% for the 1:5-lactone solution are obtained. These figures are based on the assumption that the main lactone present after 24 hr. at 100° is the 1:4-lactone (Hedenburg, 1915).

	Glucono-1:4-lactone		Glucono-1:5-lactone	
Time (hr.)	Inhibition (%)	$[\alpha]_D^{18}$ (*c*, 1·8)	Inhibition (%)	$[\alpha]_D^{18}$ (*c*, 1·8)
0	71·6	+67·0°	77·0	+63·1°
1	67·2	—	66·9	—
2	67·3	—	66·8	—
3	66·7	—	66·2	—
4	66·9	—	66·7	—
5	67·0	—	66·8	—
17	66·9	—	67·0	—
24	66·8	+40·9°	66·7	+38·0°

inhibition to be due to the 1:4-lactone) are in good agreement with those calculated from the observed rotation. Gluconic acid on heating at 100° would be expected to attain the same equilibrium, and though on heating a solution of sodium gluconate with an equivalent of HCl at 100° for 24 hr. neither the percentage inhibition nor the rotation was as high as that obtained on heating the lactones, there was, nevertheless, a notable increase in inhibitory power, and agreement with the rotation. A millimolar solution of sodium gluconate, which before heating with HCl caused 4·9% inhibition, after 24 hr. heating at 100° caused 60·8% inhibition and had $[\alpha]_D^{18}$ +23·1° in water (*c*, 1·8, calculated as lactone). Presumably equilibrium had not yet been finally established. Early attempts to obtain solutions of the 1:4-lactone by heating sodium gluconate solutions with HCl showed little or no increase in inhibition, and led to a false conclusion regarding the inhibitory power of the 1:4-lactone published in a preliminary communication (Conchie, 1953).

DISCUSSION

Of the total β-glucosidase activity demonstrated to be present in the rumen by the use of *o*-nitrophenyl β-D-glucoside, only the enzyme in the larger particles has been studied in detail, though preliminary observations on the remaining fraction indicate that it has similar properties. By neglecting this fraction for the time being, larger quantities of rumen liquor could be dealt with. Moreover, the fractions examined contain by far the greater proportion of the total activity.

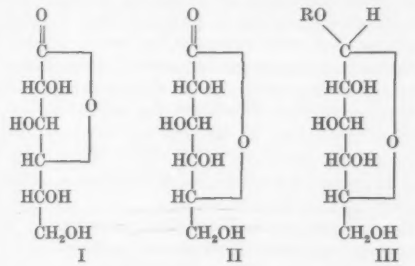
Criticism of the use of *p*-nitrophenyl β-D-glucoside as a means of estimating total enzyme activity has come from Jermyn (1952). After applying the techniques of filter-paper electrophoresis and paper chromatography to test the homogeneity of β-glucosidases from *Aspergillus oryzae* he found that the preparation consisted of a number of components, none of which had absolute specificity for any one substrate. On the other hand, only one of these components would hydrolyse *p*-nitrophenyl β-D-glucoside, and this appeared to be part of a separate system, not necessarily related to the other β-glucosidases. Niwa (1943), investigating the specificity of β-glucosidase preparations from animal viscera, studied the activities of preparations obtained under varying conditions from ox liver, and found that while the activity on salicin always varied parallel to that on phenyl β-glucoside, the activities on *p*-nitrophenyl and β-naphthyl β-glucosides varied irregularly. In the case of horse-kidney β-glucosidase, he obtained a different pH optimum using *p*-nitrophenyl β-glucoside compared with that obtained using salicin and phenyl β-glucoside.

While it is true that, in some cases at least, the nitrophenol colorimetric method does not seem to indicate all the β-glucosidase activity present in a preparation, it is nevertheless useful in a preliminary survey. The above evidence merely emphasizes that it would be unwise to carry out too extensive a study of the enzyme relying solely on this method of assay. An indication that the preparation discussed in this paper is not solely an aryl β-glucosidase, but also capable of hydrolysing cellobiose, is given by the fact that a 10 mM solution of cellobiose causes about 30% inhibition of the hydrolysis of *o*-nitrophenyl β-D-glucoside. It was considered inadvisable to carry out measurements of the liberation of reducing sugar until some further purification of the preparation had been done. This is in progress.

Until recently it was considered that particles in rumen liquor brought down at relatively high centrifuge speeds consisted essentially of micro-organisms of various types. However, Davidson (1954) has shown by measurement of their plant pigment content, that all centrifugal fractions in rumen liquor, even those composed of the smallest particles, contain plant fragments derived from the diet. In the present connexion, this introduced the possibility that plant enzyme might contribute to the total β-glucosidase activity in the rumen fractions. Accordingly, investigations on the β-glucosidase activity of the hay fed to the sheep were carried out.

A whole suspension of hay showed β-glucosidase activity, a large proportion (63%) of which could be removed by washing with 1% NaCl solution. Only a very small proportion of the enzyme activity in an acetone-treated powder prepared from the hay suspension could be extracted with an 0·05% (w/v) solution of Triton X-100. Since the enzyme in the fractions prepared from rumen liquor by centrifuging was not extracted by 1% NaCl solution, but was largely extractable, after acetone treatment, by 0·05% Triton X-100, it would appear to be of microbial origin.

In view of the fact that the β-glucosidase activity of hay could be extracted with 1% NaCl solution, it is remarkable that the supernatant obtained after freeing rumen liquor from all particulate matter showed no β-glucosidase activity, since the β-glucosidase present in the rumen is stable at the pH of rumen liquor. This absence of activity in the supernatant must mean that any β-glucosidase present in the hay eaten by the sheep is rapidly adsorbed, absorbed or metabolized by the rumen micro-organisms as soon as it goes into solution.



Levvy (1952), studying inhibitors of β-glucuronidase, found that while saccharo-1:4-lactone was an inhibitor, saccharic acid was not, and suggested that this was due to the fact that the general formula for a β-D-glucuronide more closely resembled that for saccharo-1:4-lactone than saccharic acid, having a ring system involving $C_{(1)}$ and a carboxyl group on $C_{(6)}$. A similar comparison might be made in the case of β-D-glucosides (III) and the lactones of gluconic acid (I and II). Since both lactones have about the same degree of affinity for the enzyme, it would appear that, while a ring system is necessary, it can involve $C_{(4)}$ or $C_{(5)}$ without appreciable difference in effect. Saccharo-1:4-lactone had no inhibitory effect on rumen β-glucosidase, so a primary alcohol group on $C_{(6)}$ is probably a requisite for inhibition of this enzyme.

Ezaki (1940), investigating the β-glucosidases of takadiastase and almond emulsin, found that gluconic acid, and its 1:4-lactone, were inhibitors of β-glucosidase activity in takadiastase, but that gluconic acid did not inhibit the β-glucosidase activity of emulsin. This work was further extended by Horikoshi (1942), who found that the β-glucosidase of emulsin was inhibited by glucono-1:4-lactone. On the basis of inhibitor studies these authors found that β-glucosidase preparations from various sources were of two types—the 'emulsin' type or the 'taka' type. Neither author appears to have tested the 1:5-lactone.

The most interesting feature of the inhibition experiments described in this paper is the prospect that not only β-glucosidases, but β-glycosidases and possibly glycosidases are in general inhibited by the aldonolactones of configuration corresponding in the secondary hydroxyl groups to the sugar residues and with identical groups at $C_{(6)}$.

SUMMARY

1. A β-glucosidase preparation, essentially microbial in origin, has been obtained as a cell-free extract from sheep rumen contents, and its properties have been investigated using *o*-nitrophenyl β-D-glucoside as substrate.

2. The preparation has two pH optima, at pH 5·4 and pH 5·8 respectively.

3. A value of 0·86 mM was obtained for K_m, the dissociation constant of the enzyme–substrate complex, for *o*-nitrophenyl β-D-glucoside.

4. The enzyme is stable between pH 5 and 7, and is stable at the temperature of assay, 37°, for 4 hr.

5. Both glucono-1:4-lactone and glucono-1:5-lactone are strong competitive inhibitors of rumen β-glucosidase activity, having an affinity for the enzyme about 10 times that of *o*-nitrophenyl β-D-glucoside. Gluconic acid has no appreciable inhibitory effect.

6. The changes in inhibitory power of the two lactones in aqueous solutions at room temperature and 100° can be correlated with the structural changes occurring in the lactones.

The author wishes to express his thanks to Dr G. A. Levvy for his interest and encouragement, and for his suggestion of gluconolactone as a possible inhibitor, to Dr C. A. Marsh for the sample of thiophenyl β-glucoside, and to Miss E. F. Wallace and Mr I. Strachan for technical assistance.

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The Action Pattern of Potato Phosphorylase

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(*Received* 15 *April* 1954)

The action of phosphorylase is expressed by the equation

$$n\text{ (glucose 1-phosphate)} + \text{primer} \rightleftharpoons \text{amylose} + n\text{ (orthophosphate)}.$$

The enzyme belongs to the group of carbohydrate-transferring enzymes, the function of which is the transfer of sugar residues from the donor substrate (in this case, glucose 1-phosphate or amylose), either singly or severally to an acceptor substrate containing a hydroxyl group (primer or orthophosphate).

The present investigation is concerned with two problems which are common to these transferring enzymes. The main problem is the elucidation of the mode of action of the enzyme, but this is bound up with the problem of the function of the maltodextrins (maltosaccharides) in the forward (synthetic) reaction. Confining the attention to this synthetic reaction two patterns of action may be envisaged. These are described as 'single-chain' and 'multi-chain'. In the former, a molecule of phosphorylase continuously increases the length of a single primer chain until the molecule becomes immune to further enzyme action (e.g. by precipitating from solution), before transferring its activity to a second molecule of primer. In the multi-chain type of action random synthesis occurs and all the primer chains grow at approximately equal rates. This seems the more probable mechanism and has generally been assumed in the past (e.g. see Swanson, 1948); Bernfeld & Meutémédian (1948), however, favoured single-chain action. If it could be shown that multi-chain synthesis occurred, then by the use of primer molecules of known size, the synthesis of linear chain molecules of any predetermined average length could be accomplished. These substances would provide the material for studies of the variation in physical and chemical properties in a homologous series of polymers and of the action patterns of other enzymes. In this paper it is established that synthesis by phosphorylase follows the multi-chain pattern, and synthetic amyloses have been prepared for use in studies of variation in iodine stain with chain length (Whelan & Bailey, to be published) and of the action patterns of Q-enzyme (Peat, Whelan & Bailey, 1953) and β-amylase (Whelan & Bailey, to be published). Through the initiative of Mould & Synge (1954) they have also been used in developing a new method of fractionating large molecules.

The second problem required a study of the behaviour of maltodextrins as primers in the synthetic reaction. Hanes (1940) first showed that the synthetic reaction did not proceed in the absence of starch, dextrins or maltose. Green & Stumpf (1942) failed to detect priming action with maltose but confirmed Hanes's other observations. Weibull & Tiselius (1945) hydrolysed a starch dextrin (produced amylolytically) with acid and fractionated the products on charcoal using aqueous ephedrine as eluting agent. They found that the product of lowest molecular weight to exhibit priming activity was the trisaccharide, maltotriose. We have re-investigated the priming activity of the maltodextrins using material prepared as described by Whelan, Bailey & Roberts (1953). It will be shown that the choice of primer can exercise a profound influence on the polydispersity of the synthetic amylose; if maltotriose had been the only primer used in the study of action pattern the conclusion might well have been that single-chain synthesis was taking place. Maltotriose is unique among the maltodextrins in respect of the action on it of starch-metabolizing enzymes. It is very slowly attacked by β-amylase (Whelan *et al.* 1953)

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and by crude salivary α-amylase (Whelan & Roberts, 1953) and was found to be a relatively very inefficient primer in amylose synthesis (Bailey, Whelan & Peat, 1950). This last property has been the subject of a detailed study which is also reported below.

Some aspects of this work have been summarized by Bailey *et al.* (1950) and Bailey & Whelan (1952).

MATERIALS AND METHODS

α-D-Glucose 1-phosphate. The primer-free dipotassium salt was prepared as by McCready & Hassid (1944), and used as a 0·1 M aqueous solution, adjusted to pH 7·0 with H_2SO_4.

Maltodextrins. These were prepared from potato amylose and the concentrations of their aqueous solutions determined as described by Whelan *et al.* (1953).

Achroic primer. Potato amylose (100 mg.) was dissolved in N-NaOH (10 ml.) and acidified with N-H_2SO_4 (12 ml.) giving a solution 0·1 N with respect to acid. This was heated for 2 hr. at 100°, when the iodine stain was found to have disappeared. The solution was then cooled, neutralized (NaOH), diluted to 100 ml. and stored under toluene.

Buffers. Unless otherwise mentioned, all buffers were 0·2 M in concentration and were constituted as follows: citrate (citric acid, NaOH; pH 7·0), phosphate (KH_2PO_4–Na_2HPO_4; pH 6·7) and acetate (acetic acid–NaOH; pH 4·8 and 7·0).

Iodine solution. All iodine staining was carried out with 0·2% (w/v) I_2 in 2% (w/v) KI.

α-Amylase. This was prepared from human saliva as by Whelan & Roberts (1953). The method is essentially that of Meyer, Fischer, Staub & Bernfeld (1948).

β-Amylase. A preparation was obtained from soya beans as described by Peat, Pirt & Whelan (1952*b*) and a specimen of the crystalline enzyme from sweet potato was kindly provided by Dr A. K. Balls. Both preparations exhibited the same properties (Peat, Pirt & Whelan, 1952*a*, *b*) and were used interchangeably. The enzyme activity was determined as by Hobson, Whelan & Peat (1950).

Phosphorylase. The enzyme was obtained from potatoes (3·5 kg.) following the method of Barker, Bourne, Wilkinson & Peat (1950) as far as the elution of the lead complex. The protein was then fractionated with 50% (w/v) $(NH_4)_2SO_4$ solution (pH 7·0; 0°) by rejecting the fraction precipitating at 19% (w/v) $(NH_4)_2SO_4$ and precipitating the phosphorylase at 35% (w/v). This was redissolved in water and the procedure repeated by rejecting the further precipitates obtained at 21, 23 and 25% (w/v). The enzyme was precipitated with 35% (w/v) $(NH_4)_2SO_4$ after each of these steps. Centrifuging was carried out at room temperature. The final precipitate was dissolved in 0·5 M sodium citrate buffer (pH 6·0; 75 ml.) and freeze-dried. In a typical preparation the resulting powder (10 g.) had an activity of 7·5 units/g. (see below).

Determination of inorganic phosphate. The method of Allen (1940) was used. In this method the molybdenum blue colour is developed with a mixture of 60% (w/v) $HClO_4$, 1% (w/v) amidol (2:4-diaminophenol hydrochloride) in 20% (w/v) $NaHSO_3$ and 8·3% (w/v) ammonium molybdate. Glucose 1-phosphate was frequently present in solutions under determination. An appreciable error resulted from hydrolysis of the phosphate ester when in contact with these reagents. An attempt was made to eliminate this error by making the measurements at exactly the same time after mixing the reagents, but failed when it was shown that the rate of hydrolysis of the ester was influenced by the amount of inorganic phosphate present. The problem was resolved by the accidental discovery that hydrolysis was very much reduced by halving the specified quantity of amidol reagent. The quantities of reagents now used were, $HClO_4$ (2 ml.), amidol (1 ml.) and molybdate (1 ml.) in a total volume of 25 ml. By this means the average increase in intensity of the blue colour between 9 and 30 min. after mixing the reagents was reduced from 4·0 to 0·6 scale units on the 'EEL' colorimeter (Evans Electroselenium Ltd.) using 1·3 cm. diam. cells and Ilford filter no. 608 (max. transmission, 680 mμ.). The average equivalent of 0·1 mg. of phosphorus in colorimeter scale units is 65.

Determination of glucose 1-phosphate. A modification of the method of Hanes (1940) was used. This employs acidic hydrolysis of the ester by heating its solution for 7 min. at 100° with the 60% (w/v) $HClO_4$ (2 ml.) in a total volume of 10 ml., cooling and adding amidol and molybdate as above and finally diluting to 25 ml. with water. Inorganic phosphate present before hydrolysis is also determined and the difference between the determinations is the measure of ester phosphate. When the amount of inorganic phosphate is so large as to make the difference determination inaccurate this ion is first removed by precipitation with magnesia mixture ($MgCl_2$, 110 g. + NH_4Cl, 140 g. + 1·65 N-NH_4OH, 2 l.; stand for 3 days and filter). Control experiments with solutions varying from 0·5 to 3·5 mM with respect to glucose 1-phosphate and up to 50 mM in inorganic phosphate showed that losses as high as 24% of ester phosphate occurred by co-precipitation. No loss occurred if the inorganic phosphate content was kept below 5 mM, by dilution if necessary, before adding magnesia reagent (0·25 vol.). Even after precipitation with magnesia reagent it was still necessary to determine inorganic phosphate present before hydrolysis, since traces of inorganic phosphate remained unprecipitated.

Determination of phosphorylase activity. Two digests containing citrate buffer (1 ml.), 1% soluble starch (0·5 ml.), enzyme solution (0·5 ml.) and water (0·5 ml.) were prepared and brought to 35° in a thermostat. To one digest was added 0·1 M glucose 1-phosphate (1 ml.), preheated to 35°. After 12 min. a portion (1 ml.) was removed and added to 60% (w/v) $HClO_4$ (2 ml.) in water (approx. 8 ml.) for determination of inorganic phosphate. At the same time the ester phosphate solution (1 ml.) was added to the second digest with shaking and a portion (1 ml.) immediately withdrawn for determination of the initial inorganic phosphate content of the digest. The unit of activity of the enzyme is defined as that amount which will cause the liberation of 0·1 mg. P in the above digest during 3 min.

Measurement of light absorption. Light-absorption curves of iodine-stained polysaccharides were measured in the Unicam SP. 500 spectrophotometer, using 1 cm. cells.

Inhibition of enzymic impurities. In some experiments traces of amylolytic enzymes, Q-enzyme and phosphoglucomutase present in the phosphorylase preparation were inhibited by the inclusion in the digest of $HgCl_2$. Phosphatase, attacking glucose 1-phosphate, was inhibited by ammonium molybdate (Bailey, Thomas & Whelan, 1951). Control experiments showed that these inhibitors did not interfere with phosphate determination nor did they influence the iodine-staining properties of synthetic amyloses.

Reducing power. This was determined with the Somogyi (1945) copper reagent.

Preparation of synthetic amyloses. Details are given of a typical preparation.

The digest contained maltohexaose ($3{\cdot}2 \times 10^{-5}$ moles), glucose 1-phosphate (60 ml.), citrate buffer (4 ml.), 8·3% (w/v) ammonium molybdate (2 ml.), 0·153 mM-$HgCl_2$ (0·6 ml.) and phosphorylase (200 mg.) in a total volume of 100 ml. The enzyme solution was added last and the solution was immediately diluted to the mark, mixed and a 0·3 ml. portion removed for inorganic phosphate determination, the enzyme being inactivated at once by running the solution into the perchloric acid. During incubation at 35°, further portions were removed for measurement of inorganic phosphate and the phosphate/time curve was extrapolated to the point at which calculation had shown that the average chain length of the synthetic amylose would be 60 glucose units. At this time (2 hr. 35 min. incubation) a 55 ml. portion was removed and immediately heated to 100° in a boiling-water bath, heating being continued for 3 min. The inorganic phosphate content of the cooled solution was determined and used to calculate the chain length of the amylose. The quantity of phosphate liberated gives the number of glucose units added to the primer molecules, initially six units in length. Division of the molar amount of added glucose by the known molar concentration of primer gives the average number of glucose units added to each primer molecule. In this particular experiment 55 units were added. The average chain length of the amylose was therefore 61. The remainder of the digest was incubated for a further 2 hr. and, after inactivating the enzyme, the average chain length was found to be 81·5. Both solutions were dialysed for 7 days against running tap water, centrifuged, and the supernatant solutions freeze-dried.

RESULTS AND DISCUSSION

The priming action of maltodextrins

Products of the partial acidic hydrolysis of amylose, i.e. glucose, maltose, maltotriose, etc., were separated by charcoal chromatography and tested, in equimolar concentration, for priming activity in the synthetic reaction. The results shown in Fig. 1 confirm previous observations (Green & Stumpf, 1942; Weibull & Tiselius, 1945) that glucose and maltose are devoid of priming activity. Maltotriose and the higher dextrins exhibit activity. Experiments were next performed to examine the relationship between the rate of phosphate liberation and the concentration of the maltodextrin primers. It became evident that the concentration of primer used in the above experiment was too high to indicate the activity of maltotriose relative to the activities of the higher dextrins. It was found that a linear relationship between these two functions existed only up to a concentration of 2×10^{-5} M for the tetra-, penta- and hexa-saccharides and up to 6×10^{-4} M for maltotriose under the experimental conditions given in Table 1 which shows the molar equivalent priming activities and demonstrates that under these conditions maltotriose has only 9% of the activity of the tetraose. Malto-pentaose and -hexaose show successively higher activities.

It was of interest to compare the activities of the maltodextrins after degradation by β-amylase in order to determine whether inferences drawn from a study of their behaviour on β-amylolysis (Whelan *et al.* 1953) could be substantiated. These earlier experiments had shown that a small amount of β-amylase, while without action on maltotriose, rapidly converted malto-tetraose, -pentaose and -hexaose into, respectively, maltose (2 mol. prop.),

Fig. 1. Priming activity of glucose and maltodextrins obtained by charcoal chromatography. Each digest except the control contained glucose or maltodextrin (1 μmole), citrate buffer (1 ml.), glucose 1-phosphate (1 ml.), phosphorylase (50 mg.) and water to 3·2 ml. During incubation at 35°, portions (0·5 ml.) were withdrawn for determination of liberated phosphate.

Table 1. *Priming activities of equimolar amounts of maltodextrins before and after β-amylolysis*

Maltodextrin (0·62 μmole maltotriose, 0·06 μmole maltotetraose, -pentaose and -hexaose) was incorporated in digests containing glucose 1-phosphate (0·2 ml.), phosphorylase (20 mg.), citrate buffer (1 ml.) and water to 3 ml. After incubation at 35° for 75 min. a portion (1 ml.) was withdrawn for determination of liberated phosphate. The same quantities of dextrin, mixed with acetate buffer (pH 4·8; 0·1 ml.), were treated with crystalline β-amylase (2·6 units) in a total volume of 1·3 ml. After 2·5 hr. at 35° the enzyme was inactivated by being heated at 100° and the above quantities of ester phosphate, buffer and enzyme were added for determination of residual priming activity. Control experiments were performed to determine phosphate liberation in the absence of added primer.

Primer	Phosphate liberated (as μg. P/μmole primer)	
	Before β-amylase action	After β-amylase action
Maltotriose	35	34
Maltotetraose	390	0
Maltopentaose	425	34
Maltohexaose	497	14

maltose and maltotriose (1 mol. prop. of each) and maltose (3 mol. prop.). High concentrations of β-amylase slowly convert maltotriose into maltose and glucose (Whelan *et al.* 1953; Whelan & Roberts, 1953). The priming activities of the products of the action of a small amount of β-amylase on the maltodextrins also confirm these conclusions (Table 1). Maltotriose activity was unaffected by β-amylolysis, that of the tetraose disappeared, pentaose activity fell to the expected triose level, and hexaose activity to 2·8% of its original level, due either to incomplete degradation to maltose or to the presence of impurities of maltopentaose and/or maltoheptaose. A further argument favouring this interpretation of β-amylase action is provided in the accompanying paper (Whelan & Roberts, 1954).

Maltotriose. Because of the relatively very low priming activity of this preparation a number of experiments were performed to test the possibility that this activity was not an intrinsic property of the triose but was due to an impurity either in the sugar or in the phosphorylase preparation. The need for such further tests was emphasized by the reasoning that the true priming activity of the triose relative to the higher dextrins is certainly much lower than that depicted in Table 1. Ideally, priming activity should be measured in terms of the rate of addition of a single glucose unit to the primer molecule. In practice, this would require greater amounts of primer than were available. However, where the rates of successive addition of several glucose units do not differ appreciably, the measurement of the overall rate of addition of many glucose units, as was being done here, provides a reasonably accurate measure of the true priming activity. In the case of an inefficient primer this measure is not accurate since the liberation of inorganic phosphate is greatly accelerated after the first addition of a glucose unit to form an efficient primer. This point is illustrated by the curve depicting maltotriose priming activity in Fig. 1. These results show that rate of reaction expressed in terms of a single measurement (the method used in Table 1) is dependent on the time of measurement and increases with time. This does not happen in the tests with the higher dextrins (Fig. 1). From a knowledge of the variation in iodine-staining properties of synthetic amyloses with chain length (Whelan & Bailey, to be published) it was found that when using maltotriose as a primer the products of synthesis accumulated in sufficient concentration to become detectable by iodine staining only when their average chain length was at least 100 units. A rough calculation based on this fact suggested that the true relative priming activities of the triose and tetraose might be in the ratio of only $1:10^4$.

The following experiments, of which only the last is reported in detail (Table 2), led us to conclude that an impurity in the maltotriose preparation, the most probable being maltotetraose, was not responsible for the priming activity.

The first series of experiments involved the action of the α- and β-amylases on the maltotriose. It had already been shown that after treatment with a small amount of β-amylase maltopentaose possessed a residual priming activity, equal on a molar basis to that of the maltotriose, while the triose itself showed undiminished activity (Table 1). These results were suggestive of the absence of a maltotetraose impurity, since this substance loses all activity when subjected to the same treatment (Table 1). Nevertheless, it could not be overlooked that maltotriose, itself very slowly attacked by β-amylase, might act as a competitive inhibitor of β-amylase and largely prevent the hydrolysis of a trace impurity. When, however, the rates of β-amylolysis of malto-tetraose or -pentaose in the presence and absence of an equimolar amount of maltotriose were compared, no effect of maltotriose on the rates of β-amylolysis of the higher dextrins was observed.

Since a small amount of β-amylase had failed to diminish the priming activity of the maltotriose preparation, an amount of enzyme sufficient to bring about the complete hydrolysis of the triose itself was next used and priming activity was measured at stages during the reaction. No initial rapid fall in activity, which would have indicated the removal of an impurity by the β-amylase, was observed; at all stages during the hydrolysis the loss of priming activity was proportional to the amount of maltotriose hydrolysed. In view of the demonstration that the addition of maltotriose had no influence on the rapid β-amylolysis of the tetraose (see above), this new result was a clear indication of the intrinsic priming activity of maltotriose. Furthermore, prolonged treatment of the maltotriose specimen with salivary α-amylase caused no detectable diminution in its priming activity. This α-amylase differs from β-amylase in having no action on maltotriose but, like the latter enzyme, converts maltotetraose into maltose (Whelan & Roberts, 1953).

When it was found that refractionation of the triose specimen on charcoal did not diminish its priming activity, a final test for the presence of a priming impurity was applied. Two digests were prepared containing phosphorylase, glucose 1-phosphate together with, in one case, maltotriose and in the other, the equivalent amount of maltotetraose in terms of priming activity. Since the 'priming equivalent' of maltotetraose was only 9% (molar basis) of that of maltotriose (when measured as in Table 1), it is to be expected that a greater weight of amylose would be synthesized with the maltotriose primer than with the tetraose, assuming

that the chains do not grow to infinite length but that synthesis is terminated by retrogradation. The results in Table 2 show that this in fact occurred. It is further to be expected that if maltotriose is a primer, albeit a poor one, $\lambda_{max.}$ of the iodine-stained products would change very little throughout the reaction. This follows because the product of addition of one glucose residue, i.e. maltotetraose, is so much more efficient as a primer that, once formed, the tetrasaccharide primer becomes considerably extended in length during the time interval before another maltotriose molecule is involved. Experiment confirms this view; the products are blue staining throughout the reaction and $\lambda_{max.}$ changes very little, viz. from an initial value of 620 mμ. to a final value of 640 mμ. (It has been established that maltodextrin chains become blue-staining when longer than about 45 units; Whelan & Bailey, to be published).

In contrast, when maltotetraose was used as primer there occurred the normal sequence of changes in iodine stain which, as will be shown in a later section, is a criterion of multi-chain synthesis, and this despite the fact that the much smaller amount of tetraose used made difficult the recognition of the early products of synthesis.

Bernfeld & Meutémédian (1948) claimed that only blue-staining products could be detected during the phosphorylase synthesis of amylose. It can, however, be inferred that these authors were using either a preparation in which maltotriose was the only primer or, more probably, one so deficient in primer that only the blue-staining products, which also have the greatest intensity of stain per unit weight, could be detected.

The work now reported was carried out in 1950–1, at which time the existence of D-enzyme in the potato was not suspected. This enzyme is able to effect a disproportionation of maltodextrins, e.g. maltotriose into a mixture containing glucose, unchanged maltotriose, maltotetraose, -pentaose, etc. (Peat, Whelan & Rees, 1953). Clearly, the presence of this enzyme could seriously affect the course of amylose synthesis from glucose 1-phosphate and maltodextrin primer and, in particular, contamination of the phosphorylase preparation might have been responsible for the apparent priming activity of the maltotriose. None of the experiments mentioned above could have revealed such action since they were tests for the purity of the substrate and not of the enzyme preparation. D-Enzyme appears not to function by a phosphorylating mechanism so that a test for its presence could be made by incubation of the phosphorylase with maltotriose before the addition of glucose 1-phosphate. It was thus shown that synthesis proceeded at exactly the same initial slow rate as when there was no pre-incubation (Table 3).

All the above experiments lead to the conclusion that maltotriose is a primer in the synthetic reaction and confirm our original statement (Bailey *et al.* 1950). Its low activity relative to that of the higher dextrins has since been confirmed by French & Wild (1953) using an ingenious paper-chromatographic method. Weibull & Tiselius (1945) reported, on the other hand, that maltotriose, -tetraose, -pentaose and -hexaose exhibited approximately equal priming activity, but it seems possible either that the maltotriose was contaminated with higher dextrins or that the activity was not being measured with the correctly proportioned amounts of dextrin (cf. Fig. 1 and Table 1).

Action pattern of phosphorylase

Three independent methods were used in the examination of the action pattern of the enzyme.

Table 2. *Amylose synthesis using equal priming activities of maltotriose and maltotetraose*

Each digest contained glucose 1-phosphate (10 ml.), acetate buffer (pH 7·0; 5 ml.) 0·153 mM-$HgCl_2$ (1 ml.), 8% ammonium molybdate (0·55 ml.), phosphorylase (300 mg.); one digest contained maltotriose (0·93 mg.) and the other, maltotetraose (0·126 mg.), the total volume being 25 ml. During incubation at 35° portions (0·5 ml. each) were withdrawn for measurement of phosphate liberation and $\lambda_{max.}$ of the iodine-stained solution as in Fig. 3. Chain length was calculated as in the Methods section.

Maltotriose primer			Maltotetraose primer		
	Iodine stain			Iodine stain	
Chain length	$\lambda_{max.}$	Colour	Chain length	$\lambda_{max.}$	Colour
8·3	620	Green	6·2	—	Yellow
12	620	Green	10	—	Yellow
19	620	Blue-green	15	500	Red
25	—		21	520	Red
36	—		30	535	Red-purple
50	—		36	555	Purple
97	635		44	570	Blue
195	640		55	585	Blue-green
			101	618	
			164	620	

First, the kinetics of the reaction were studied in order to determine the relation between rate of amylose synthesis and primer concentration; secondly, the values of $\lambda_{max.}$ of iodine-stained amyloses were measured at stages during polysaccharide synthesis from maltotetraose primer; and thirdly, synthetic amyloses of calculated average chain length were prepared from maltohexaose primer and examined by Mould & Synge (1954) using the method of electrokinetic ultrafiltration.

Effects of variation in primer and enzyme concentrations. The rate of synthesis of amylose was measured in digests in which only the primer concentration was varied. This is the procedure followed in determining the Michaelis constant for the phosphorylase–primer complex. A linear relationship is obtained when the reciprocals of reaction velocity and primer concentration are plotted against each other (Fig. 2*a*). The same effect is shown in Fig. 2*b* where, at constant concentration of glucose 1-phosphate, the amounts of phosphorylase and primer were kept constant but the concentrations were varied by dilution. The linear relationship between the reciprocal of the reaction velocity and the square of the digest volume (Fig. 2*b*) indicates that the reaction is bimolecular with respect to enzyme and primer. Both results indicate that the primer behaves as a normal substrate in the reaction, and are consistent with a multi-chain pattern of enzyme action.

Light absorption of iodine-stained synthetic amyloses. The course of amylose synthesis from maltotetraose by the agency of phosphorylase was followed by plotting the light-absorption curves of the products stained with iodine at intervals during digestion (Fig. 3). The visual changes in colour are from brown, through red and purple to a pure blue (cf. Swanson, 1948). Since the reverse changes in colour occur when amylose is randomly fragmented by acid or by α-amylase it is clear that this pattern of absorption curves is to be associated with the regular change in length of all primer molecules. The spectral pattern of single-chain synthesis would be very different since only the final product would contribute to the light absorption. In consequence $\lambda_{max.}$ should remain constant and independent of the amount of glucose 1-phosphate consumed. The single-chain pattern was simulated, for reasons already discussed, when maltotriose was used as primer (Table 2).

It was important to determine whether, as in synthesis, phosphorolytic degradation of amylose also proceeded as a multi-chain action. This was examined by reversing the course of the synthetic reaction shown in Fig. 3 by adding an excess of inorganic phosphate to the digest. The pattern of absorption curves obtained was qualitatively the same as for synthesis but at corresponding stages in

Table 3. *Priming activity of maltotriose after pre-incubation with phosphorylase*

Three digests were prepared containing citrate buffer (1 ml.), 8·3% ammonium molybdate (0·1 ml.), 0·153 mM-$HgCl_2$ (0·1 ml.), phosphorylase (30 mg.) and maltotriose (1 mg.) in 2·8 ml. After 0, 2 and 4 hr. respectively, at 35°, glucose 1-phosphate (1·2 ml.) was added to each digest and portions (0·5 ml.) were removed for determination of liberated phosphate. Incubation was at 35°. A control digest was used to determine phosphate liberation in the absence of added primer, and further control digestions showed that phosphorylase activity did not begin to decay appreciably until after 8 hr. at 35°.

Time of incubation (hr.)	Time of pre-incubation (hr.) 0	2	4
	Phosphate liberated (colorimeter units)		
1	2·7	3·3	2·8
2	8·6	8·7	7·8
4	15·9	14·9	12·0
6	25·4	23·5	15·6
8	34·8	27·5	22·0

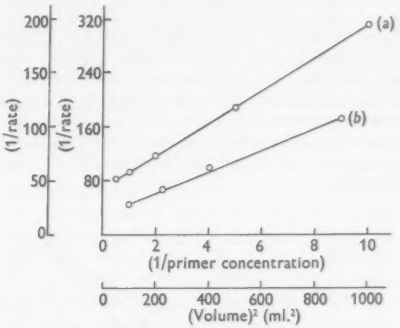

Fig. 2. (*a*) Michaelis–Menten relationship for the primer–phosphorylase system. Digests contained achroic primer (0, 0·1, 0·2, 0·5, 1·0 and 2·0 mg.), phosphorylase (100 mg.), glucose 1-phosphate (2 ml.), acetate buffer (pH 7·0; 1 ml.) and water to 6 ml. After incubation for 3 hr. at 20° portions (1 ml.) were withdrawn for measurement of phosphate liberation. Rate is expressed as mg. phosphorus liberated/min. in the whole digest and concentration as mg. primer in the digest. (*b*) Showing that at constant initial glucose 1-phosphate concentration, synthesis is bimolecular with respect to enzyme and primer. Digests contained achroic primer (1 mg.), phosphorylase (50 mg.), acetate buffer (pH 7·0; 2, 3, 4 and 6 ml.), glucose 1-phosphate (2, 3, 4 and 6 ml.) and water to 10, 15, 20 and 30 ml. respectively. During incubation at 35°, portions (0·2, 0·3, 0·4 and 0·6 ml. respectively) were withdrawn for measurement of liberated phosphate. The rate of liberation was uniform in each digest during the first 60 min. Rate is expressed as mg. phosphorus liberated/min. in the whole digest.

synthesis and degradation $\lambda_{max.}$ was higher in the latter reaction (Fig. 4). Phosphorolysis therefore differs from synthesis in giving an appearance of some degree of single-chain action. This behaviour could be shown however to be in accordance with the predictable characteristics of completely multi-chain action. During multi-chain synthesis, even from a homogeneous primer, the synthetic amylose becomes polydisperse, and increasingly so. It can be adduced on theoretical grounds that polydispersity of the product increases also when the reaction is reversed by the addition of inorganic phosphate to the digest. It will diminish as the minimum length of priming chain is approached. Only when this minimum is reached are all the chain molecules of the same length, a length (three glucose units, see above) which is well below the chain length requisite for iodine staining, estimated to be *ca.* 12 units. From a study, to be published later, of the relationship between chain length and $\lambda_{max.}$ of the iodine stain it has emerged that, provided the average chain length is less than 80 units, the $\lambda_{max.}$ values of amyloses of the same average length increase with increasing degree of polydispersity. It is known that the average chain lengths in the experiments of Fig. 4 did not exceed 80 units. It therefore follows that at corresponding values of chain length in the synthesis and phosphorolysis of amylose, the $\lambda_{max.}$ value for phosphorolysis will be the greater, because of the higher degree of polydispersity. This is precisely the effect which was observed.

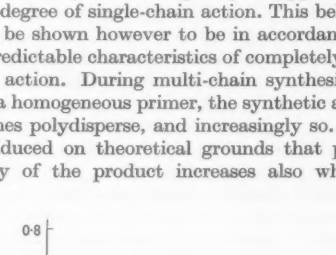

Fig. 3. Light-absorption curves of iodine-stained products during amylose synthesis from maltotetraose primer. The digest contained maltotetraose (10 mg.), glucose 1-phosphate (10 ml.), acetate buffer (pH 7·0; 10 ml.), phosphorylase (300 mg.) and water to 100 ml. At intervals during incubation at 35° portions (0·5 ml.) were stained with iodine (0·5 ml.) in a total volume of 25 ml. and their light absorption determined; further portions (0·5 ml. each) were withdrawn for measurement of liberated phosphate.

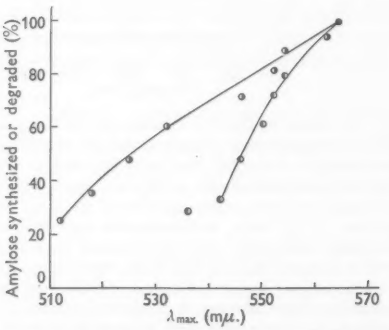

Fig. 4. Variation in $\lambda_{max.}$ of iodine-stained amylose during synthesis and degradation. Values for synthesis (◑) obtained from the experiment of Fig. 3. After incubation of this digest for 250 min. a portion (88·5 ml.) was removed and mixed with phosphate buffer (20 ml.). During further incubation at 35° portions (0·61 ml.) were removed for iodine staining as in Fig. 3 and further portions (2 ml.) for measurement of glucose 1-phosphate as in Methods section. Results represented as ◐.

A direct implication of the single-chain hypothesis is that at any time during amylose synthesis the iodine-staining material will consist of the final product, together with the relatively negligible amount of material undergoing extension of chain. In multi-chain action the properties of the product will depend on the extent to which synthesis has proceeded. Thus it follows that if in a series of syntheses the primer concentration is varied, but the reaction is arrested at stages when the amount of glucose 1-phosphate consumed in each case is the same, then the iodine-stained products will have the same or different values of $\lambda_{max.}$, according to whether single- or multi-chain action is operative. To this statement must be added the proviso that synthesis must not be allowed to proceed to the point at which the value of $\lambda_{max.}$ has become independent of chain length (Whelan & Bailey, forthcoming publication). The results of an experiment of this type (Fig. 5) are clearly in favour of multi-chain synthesis and the relative values of $\lambda_{max.}$ accord with the expectation that the greater the amount of primer present, the shorter is the average chain length of the product at stages where an equal number of glucose units has been distributed among the primer molecules. This effect has previously been noticed by Sumner, Somers & Sisler (1944).

Electrokinetic ultrafiltration of synthetic amyloses. The foregoing experiments provide strong circumstantial evidence that the enzyme action pattern is multi-chain. It was realized, however, that none of this evidence excluded the possibility that the action might not be entirely multi-chain but might also show single-chain characteristics. Such behaviour has been demonstrated in the case of β-amylase and was detected by the finding that the proportion

of each type of action could be varied by changing the temperature of incubation (French, Knapp & Pazur, 1950; Whelan & Bailey, to be published). Two series of experiments showed that similar considerations did not apply to the action of potato phosphorylase. First, the action pattern was shown not to vary with temperature (Whelan & Bailey, to be published). Secondly, a direct examination of the range of DP (degree of polymerization) in the synthetic amyloses was made by Mould & Synge (1954). (In the case of linear molecules DP is, of course, synonymous with chain length when both are expressed in terms of polymer units.) Before the latter results are considered it is profitable to consider in some detail the factors which may influence the course of multi-chain synthesis.

It is obvious that in any polymer synthesized by the endwise apposition of polymer units to priming molecules the range of DP of the chain molecules must increase as the average molecular weight is increased. Reference is being made here and throughout the paper to the absolute range of DP. As pointed out by Flory (1940) the range decreases with increase in DP if expressed as a percentage of mean DP of the preparation (see Table 4). The increase in absolute range of DP occurs even if the addition of the units is completely random and statistical calculation of this range can be made at any stage during such synthesis (Table 4). However, a number of factors may operate to increase the range. These are as follows:

(1) Table 1 shows that the priming efficiency of maltodextrins increases with molecular size in the series from maltotriose to maltohexaose and it cannot be assumed that the activity of maltohexaose is the maximum possible. The effect on the range of DP of the synthetic amylose of using a primer of submaximal activity has been mentioned above in comparing the products synthesized when malto-triose and -tetraose are used as primers. This same effect, although much less serious than in the case of maltotriose, must influence the range of DP in the synthetic amyloses which we prepared from maltohexaose for examination by Mould & Synge (1954). In the absence of knowledge of the priming activity of maltodextrins of greater molecular weight than the hexaose, no estimate of the magnitude of this effect can be made. It is possible, however, that priming activity passes through a maximum and begins to diminish with subsequent increase in chain length. Such an effect would operate to decrease the range of DP.

(2) The turnover number of potato phosphorylase has not been determined; that of muscle phosphorylase is 4×10^4 linkages formed per minute in the synthetic reaction at pH 6·7 and 30° (Cori, Cori & Green, 1943). If the potato enzyme has the same high rate of action the possibility must be considered that once contact has been established between enzyme and primer the latter may not be able to diffuse away after the apposition of one glucose unit but may undergo further chain lengthening. Such multiple attack (a term introduced by French & Wild, 1953) must occur even though synthesis may be completely random and would be favoured by a high rate of enzyme action. Again, no information on this aspect is available except that, as mentioned above, the effect is certainly operative during β-amylolysis and varies with temperature (the turnover number of β-amylase is $2{\cdot}5 \times 10^5$; Englard & Singer, 1950; Englard, Sorof & Singer, 1951), but the pattern of phosphorylase action on amylose molecules up to an average chain length of 49 units does not vary with temperature (Whelan & Bailey, to be published).

(3) Naturally occurring amyloses show a marked tendency to retrograde from solution and this also begins to occur during the synthesis of amylose when the average chain length becomes greater than about 200 units. The process is gradual and does not appear to terminate the length of every chain at the same limit. The extent of retrogradation is to some degree controlled by the amylose concentration and by the other solutes present. It may not always be

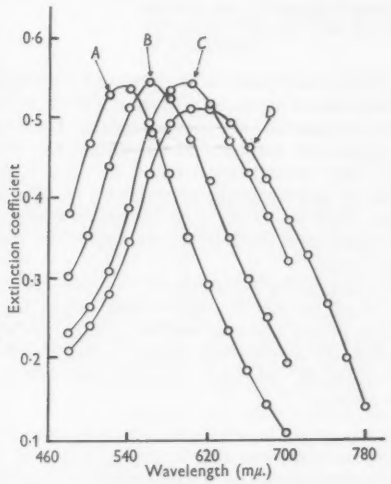

Fig. 5. Light-absorption curves of iodine stains of equal weights of amylose, synthesized from varying amounts of primer. The digests, corresponding to curves *A*, *B*, *C* and *D*, contained glucose 1-phosphate (1 ml.), phosphorylase (15 mg.) and achroic primer (2, 1, 0·5 and 0·2 mg. respectively) in 10 ml. Liberation of phosphate was followed during incubation at 35° and when, in each digest, 0·46 mg. of phosphate had been liberated, portions (2 ml.) were withdrawn and stained with iodine (0·5 ml.) in a total volume of 25 ml.

Table 4. *Comparison of experimentally determined and calculated ranges of DP in synthetic amyloses*

Calculated values were obtained by Boyne (see text) and the experimental values were determined by Mould & Synge (1954). In each case the central range of chain length (DP) estimated to contain 95% of the polysaccharide molecules is given.

	Degree of polymerization (glucose units)						
Average chain length	34	47·5	77	103	160	205	250
Experimental range	23–46	35–62	64–89	80–124	150–170	140–250	170–320
Calculated range	23–45	34–61	60–94	83–123	135–185	177–233	219–281

evident in the form of a visible precipitate but it may be assumed that a retrograded molecule is no longer a primer. When retrogradation begins to occur, therefore, the range of DP of the amylose molecules will begin increasingly to exceed the calculated range.

These three effects would tend to increase the range of DP in the synthetic amylose as compared with the range expected from normal kinetic considerations. The opportunity of testing their influence was provided by Mould & Synge (1954), who examined a series of synthetic amyloses by electrokinetic ultrafiltration and provided estimates of their DP ranges for comparison with the calculated values. The latter values were calculated by Mr A. W. Boyne of the Rowett Research Institute assuming completely random multi-chain action and no variation in priming activity with increase in chain length. These assumptions lead to a Poisson distribution of chain lengths, readily permitting the determination of the central range of DP containing 95% of the polysaccharide molecules (cf. Flory, 1940). The results for a series of synthetic amyloses, ranging in average chain length from 34 to 250 glucose units, are given in Table 4.

The finding of Mould & Synge (1954) that the mobility of a synthetic amylose in collodion is a function of its calculated average chain length provides a direct confirmation of the multi-chain nature of phosphorylase action. The results of their experiments with a series of synthetic amyloses are given in Table 4 together with the corresponding calculated ranges of DP. There is a striking agreement between calculation and observation of range of DP in the region between average DP 34 and average DP 160. Clearly none of the factors discussed above has any noticeable effect on the composition of an amylose preparation having an average DP within this range. Above DP 160 such effects are beginning to operate; the action pattern is beginning to change, not because of a change in the behaviour of the enzyme, but because of the changing properties of the primer substrate.

It is of interest to compare the results of Table 4 with the electrokinetic ultrafiltration analysis of a polysaccharide of calculated average DP 75·5 synthesized from a heterogeneous primer. This analysis and the preparation of the primer is reported in an accompanying paper (Mould & Synge, 1954) where it is shown that the actual DP range of the polysaccharide was far removed from the calculated DP, being 110–200 with a predominance of the higher polymers. The explanation of the difference between the two values for DP is similar to that given above in connexion with the properties of the amyloses synthesized from maltotriose and will not be enlarged upon here except to point out that the use of an inhomogeneous primer in preparing synthetic amyloses obviously leads to erroneous estimates of DP when calculation is made in terms of phosphate liberation. For this reason the results of Swanson (1948), where the iodine stain/chain length relationship of synthetic amyloses is reported, are in error.

SUMMARY

1. The action pattern of potato phosphorylase has been shown to be completely multi-chain both in the synthesis and the phosphorolysis of amylose.

2. Synthetic amyloses of known average chain length have been prepared and up to 160 their degrees of polydispersity (determined by Mould & Synge, 1954) are shown to be entirely in agreement with values calculated on the assumption of multi-chain action.

3. The priming activities of the maltodextrins have been compared. Maltotriose is the lowest member of the series to exhibit priming activity. This activity is very weak and the higher members, malto-tetraose, -pentaose and -hexaose are much more efficient.

We gratefully acknowledge the advice and encouragement given by Professor Stanley Peat, F.R.S. One of us (J. M. B.) thanks the Department of Scientific and Industrial Research for the award of a maintenance grant. We also thank Drs D. L. Mould and R. L. M. Synge and Mr A. W. Boyne for providing the data in Table 4.

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Products of the β-Amylolysis of Maltodextrins

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(*Received* 15 *April* 1954)

β-Amylase degrades α-1:4-linked polymers of D-glucopyranose by an end-wise action, commencing at the non-reducing chain end, in which alternate linkages are hydrolysed and maltose is liberated in the β-form (Kuhn, 1925; Hanes, 1937; Hestrin, 1949; Bird & Hopkins, 1954). In the linear series of polymers based on maltose (maltodextrins) the smallest molecule which undergoes rapid hydrolysis is maltotetraose (Whelan, Bailey & Roberts, 1953). Maltotriose is attacked very slowly with the production of glucose and maltose. From a study of changes in reducing power during hydrolysis (Whelan *et al.* 1953), and of the priming action in the enzymic synthesis of amylose after treatment of the maltodextrins with β-amylase (Whelan & Bailey, 1954), it was inferred that the rapid β-amylolysis proceeded no further than the conversion of maltotetraose and -hexaose into maltose, and of maltopentaose into equimolar amounts of maltose and maltotriose. The present work affords conclusive proof of the correctness of these deductions by the isolation and characterization of these products.

EXPERIMENTAL AND RESULTS

Maltodextrins. These were prepared from potato amylose as by Whelan *et al.* (1953).

β-Amylase. The crystalline enzyme from sweet potato was used. This was kindly provided by Dr A. K. Balls. Its activity was determined as by Hobson, Whelan & Peat (1950).

Sugar acetates. These were prepared and the maltotriose acetate was purified on Magnesol–Celite as described by Whelan & Roberts (1953).

β-Amylolysis of maltodextrins. The maltodextrin (*ca.* 100 mg.) was dissolved in water (10 ml.) and Somogyi's (1945*b*) deproteinizing reagents, 5% (w/v) $ZnSO_4$ (2 ml.) + equiv. amount of 0·4 N-$Ba(OH)_2$, added (cf. Whelan *et al.* 1953). The supernatant solution was divided into a 1 ml. portion used to determine the maltodextrin concentration (Pirt & Whelan, 1951) and a 10 ml. portion to which was added 0·2 M sodium acetate–acetic acid buffer (pH 4·8; 6 ml.), β-amylase (60 units; 1 ml.) and water to 50 ml. Measurements of reducing power were made on 1 ml. portions at intervals during incubation at 35° using the Somogyi (1945*a*) copper reagent. The hydrolyses were virtually complete after 30 min. but digestion was allowed

to continue for a total of 21 hr. At this time the percentage conversions of malto-tetraose and -hexaose into maltose were 94·1 and 95·3% respectively; the reducing power of the maltopentaose digest, calculated as an equimolar mixture of maltose and maltotriose, corresponded to a conversion of 99·8%. The enzyme was inactivated by being heated at 100° for 5 min. and the remainder of each digest (*ca.* 42 ml.) was added to a charcoal–Celite column in which the dimensions of the adsorbent were 55 × 2·0 cm. The columns were washed with water (1 l.) to remove buffer salts. Glucose would also have been removed had it been present but none was detected. The di- and tri-saccharide components were eluted with 7·5 and 15% (v/v) aqueous ethanol respectively, as described by Whelan *et al.* (1953). No trisaccharide products were obtained from malto-tetraose and -hexaose but in all three cases a small amount of optically active material ($\alpha = ca.$ 0·1° in 100 ml. solution; 4 dm. tube) was eluted when the ethanol concentration was increased from 15 to 50%. The same amount of this material was obtained when a control column, containing no added sugar, was similarly eluted. Paper chromatographic analysis of the fractions eluted with 50% ethanol showed them to contain substances reacting with the ninhydrin reagent and sugars of varying R_F values. In the case of the control column the sugar content after acid hydrolysis (Pirt & Whelan, 1951) corresponded to 1·6 mg. (as glucose) in the whole fraction (weight, 14 mg.).

The appropriate fractions containing the disaccharides were combined, as were those containing the trisaccharide. All solutions were evaporated to dryness under diminished pressure and the products acetylated. Each sample of β-maltose octa-acetate from the three maltodextrins melted (over 1°) in the range 157–160° and had $[\alpha]_D^{17}$ in $CHCl_3$ in the range 63·5–65·0 ± 2°. The yields in the acetylation of the maltose specimens varied from 58 to 100%. Authentic octaacetyl β-maltose had m.p. 159–160° and $[\alpha]_D^{17}$ +63° in $CHCl_3$. The β-maltotriose undeca-acetate (yield, 75%) had m.p. 134·5–136·5° and $[\alpha]_D^{17}$ +89 ± 3° in $CHCl_3$. The corresponding values for an authentic specimen were 134–136° and +86°.

DISCUSSION

The results given above prove that the previous deductions as to the course of β-amylase action on the maltodextrins were correct. So far as we are aware this is the first report of a complete chemical identification of all the products of β-amylase action on linear substrates. Amylose and amylopectin, as isolated from starch, have not yet proved amenable to such accounting because of the presence of branch linkages additional to the chain-forming α-1:4 linkage. This evidence of the course of β-amylase action has provided justification for an enzymic method of determining the average chain length of linear or, if a debranching enzyme is available, branched starch-type polymers based on the quantitative isolation after β-amylolysis of maltose and maltotriose, the latter being a residue from chains containing an odd number of glucose residues. Using R-enzyme in conjunction with β-amylase this method has been used in a determination of the average repeating chain length of waxy maize starch (Thomas, 1952; see Whelan, 1953), and the result was in close agreement with the value obtained by periodate oxidation.

SUMMARY

The β-amylolysis of three maltodextrins has been studied and the products isolated by charcoal chromatography. The yields and the nature of the products are in accordance with the postulated course of action of β-amylase.

The advice and encouragement given by Professor Stanley Peat, F.R.S., are gratefully acknowledged. The authors are also grateful to the Brewing Industry Research Foundation for a grant to one of them (J.G.R.).

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Separations of Polysaccharides related to Starch by Electrokinetic Ultrafiltration in Collodion Membranes

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(*Received* 15 *April* 1954)

For a series of 'isochemical' molecules, e.g. mixtures of polymeric chain molecules differing only in degree of polymerization (DP) and not otherwise in chemical nature (cf. Brønsted, 1931), the distribution coefficient between two phases at equilibrium changes by a constant factor for each unit added to the molecule. This is the basis for Traube's Rule and for the success of both adsorption and partition chromatography in separating homologues and the lower members of polymeric series (cf. Martin, 1949). However, with larger molecules in such series these methods have not proved successful. Methods used for fractionating polymeric materials have been reviewed by Cragg & Hammerschlag (1946). With the larger molecules it becomes difficult, as Brønsted showed, to find two-phase systems not giving distribution coefficients that are excessively high or low. But there is an additional difficulty due to the porous nature of many of the materials used for chromatography. With such materials as charcoal, silica gel, starch, cellulose or ion-exchange resins, whilst the affinity of the active surfaces or sorbed liquid is greater for the larger molecules, these are often prevented from penetrating the grain or fibre by molecular-sieve effects. Thus, it is usual to find that adsorption affinities increase, reach a maximum and then decrease as a polymeric series is ascended. With increasing DP, slow attainment of equilibrium and irreversibility of adsorption are also increasingly common. A number of examples of such effects in the literature have been referred to by Mould & Synge (1952); see also Deuel, Solms, Anyas-Weisz & Huber (1951), Jenckel & Rumbach (1951), Zamenhof & Chargaff (1951). If for a column consisting of separate pieces of porous material a continuous block of material of rather uniform porosity could be substituted, molecular-sieve and adsorption effects would work together and no longer in opposite senses when liquid containing molecules of different sizes is forced through the block chromatographically. For porous materials at present available, however, the high hydrostatic pressures required to produce reasonable flow through a thick block would cause stresses which would collapse the pore structure. However, electrokinetically promoted flow of liquid (electroendosmosis) does not involve applying external stresses to the porous structure. These ideas for fractionating larger molecules were discussed for some time by Dr A. J. P. Martin, Professor A. Tiselius and one of us (R. L. M. S.); the suggestion to use electrokinetic flow was prompted by observations on electroendosmotic effects during zone electrophoresis in gels (cf. Synge, 1949; Tiselius, 1949; Tiselius & Flodin, 1953). Schmid (1952) has briefly discussed the possibility of fractionating neutral molecules during electroendosmosis in ion-exchange resins, but without reference to separation according to molecular size or to the need for having the porous material in a single block.

The mixture of linear-chain molecules of different lengths obtained by partial acid hydrolysis of amylose seemed a suitable test mixture for evaluating electrokinetic ultrafiltration procedures, since complications due to differences in charge and chemical nature, such as would arise with the partial hydrolysis products of proteins, would be absent and differences in behaviour would be due to differences in molecular size only. Further, the iodine-staining characteristics of such dextrins depend on molecular size. Early experiments in which hydrolysates of amylose were subjected to zone electrophoresis in agar jelly in the presence of iodine–iodide (intended simply as an indicator) were very encouraging, since a beautiful series of coloured zones was seen to migrate towards the cathode at rates slower than that of the electroendosmosis in the jelly. The blue zones migrated more slowly than the pink and orange zones, presumed due to smaller molecules. This was demonstrated to the Biochemical Society (Synge & Tiselius, 1950). After the published abstract of this demonstration had been submitted, experiments with a different batch of agar showed equally good separation of the zones but most of the material migrated towards the anode, the blue zones migrating fastest. This phenomenon was also demonstrated to the Society and demanded a reassessment of the physicochemical basis of the separation. It became clear during subsequent experiments here and at Uppsala that the basis of the separation observed was not electrokinetic ultrafiltration but electrophoresis of complexes of the polysaccharides with negatively charged triiodide, etc. On omitting iodine, all the

polysaccharide zones moved through the agar at rates approximating to the electroendosmotic flow towards the cathode, which was much greater with the first batch of agar than with the second. However, the zones, as revealed by spraying the jelly with iodine after the run, were not quite devoid of separation; the material of high molecular weight was slightly retarded relative to that of lower molecular weight (Synge & Tiselius, unpublished observations).

The electrophoretic separations based on combination of the dextrins with iodine–iodide proved capable of preparative application and have thrown some light on the nature of the combination of starch and its breakdown products with iodine. Our experiments with these phenomena are described in the two following papers (Mould & Synge, 1954; Mould, 1954).

However, partly in view of the interest of colleagues at this Institute (especially the late Francis Baker) in the microbial breakdown of polysaccharides and in the significance of intermediates in the breakdown of starch, cellulose, etc., for the nutrition of micro-organisms, we tried also to intensify the effects noted by Synge & Tiselius in agar in the absence of iodine, which seemed possibly due to ultrafiltration retardation. Gordon, Keil, Šebesta, Knessl & Šorm (1950; cf. Gordon, Keil & Šebesta, 1949) had shown that a protein of such high molecular weight as haemocyanin can migrate electrophoretically without much hindrance in agar jelly. We accordingly tried to prepare denser gels from electrically charged materials. Experiments using gels made from polymerized formaldehyde–phenolsulphonic acid or glycerol–citric acid mixtures and gels made from carboxymethylcelluloses were only partially successful. However, promising results were obtained when collodion ultrafiltration membranes of graded porosity were used, and we obtained valuable advice about the preparation of these from Dr W. J. Elford of the National Institute for Medical Research shortly before his death.

The present paper describes experiments using electrokinetic ultrafiltration in collodion membranes. Interpretation of these and of the electrophoretic phenomena described in the following paper was greatly helped by being able to study materials by the two methods in parallel. Demonstrations of them have been given to the Biochemical Society (Mould & Synge, 1951). However, it only became possible to assess our results with any exactness when we later received from colleagues at Bangor samples of linear dextrins synthesized by the action of potato phosphorylase in digests containing glucose 1-phosphate and pure oligosaccharide primers such as maltohexaose and maltoheptaose. The products of this reaction are mixtures of dextrins grouped as to DP fairly compactly around an average which is known from the amount of inorganic phosphate liberated at the time when the synthetic reaction is stopped (Whelan & Bailey, 1954; cf. Bailey & Whelan, 1952). These reference dextrins made it possible to assess the resolving power of the procedure (while at the same time we obtained some information about the heterogeneity of the dextrins themselves), to investigate the physicochemical mechanisms on which the separations depend and to interpret observations made on various natural products related to starch and on derived materials obtained from them by enzymic or chemical treatment.

The present procedure differs in most respects from the 'electro-ultrafiltration' of Bechhold & König which has mainly been used for controlling hydrostatically the electroendosmotic flow across membranes during electrodialysis (see Bechhold, 1929; Stauffer, 1950).

We gave a preliminary account of some of the present work at the International Congress on Analytical Chemistry held at Oxford in September 1952 (Mould & Synge, 1952).

EXPERIMENTAL

Collodion membranes

Preparation. This followed in general the principles given by Elford (1931, 1938). Membranes were cast in a rectangular trough (14 × 52 cm.; 0·6 cm. deep) made by clamping together pieces of plate glass. The starting material was the ethanol–ether solution 'Necol collodion solution 301–261 (356/A9)' as sold by British Drug Houses Ltd., Poole, Dorset. After dilution with the appropriate solvents the collodion solution was poured into the trough and evaporation was allowed to proceed, without special control of room humidity or temperature, until a gel of suitable consistency was obtained. Water was then poured on and the gel was transferred from the trough to a large volume of distilled water, in which it was kept for 24 hr.; the resulting membrane was then transferred to 0·2N acetic acid, under which it was stored until required. Portions were cut from the membranes as required, rejecting parts within 1 cm. of the original edges. The mixtures used for preparing membranes for this work are shown in Table 1.

Characterization. The finer-pored membranes were tough and translucent; the coarser the pores the more opaque and brittle were the membranes. In general the properties of membranes did not change during storage under 0·2N acetic acid for a few months, but some membranes made with mixtures intermediate between those for I and III, although at first giving useful results, after about a week became coarser-pored and lacking in resolving power, suggesting that some change of structure had occurred. Some of the data given below are suggestive of a structural difference between membranes I and III, and it may well be that in membranes cast with solvents of intermediate composition the colloidal phases are in a critical state giving rise to unstable structures (see Elford, 1930). Membrane II is included as an example from this range—although made

from the same solvent mixture as the satisfactory membrane III it appeared to be coarser-pored (perhaps due to difference in the stage of gel formation at which gel was transferred to water) and never at any period during ageing gave compact, well-resolved zones with any of the dextrins tested.

The 'average pore radius' of the membranes was assessed by the rate of passage of 0·2N acetic acid at 25° through a circle of membrane 0·8 cm. in effective diameter, using the method and formula given by Elford (1931, 1938). Thickness of membranes was determined with a screw micrometer. Dry matter was determined on portions of membrane that had been quickly blotted with filter paper and weighed; these were dried to constant weight over H_2SO_4 *in vacuo* at room temperature. The viscosity of 0·2N acetic acid at 25° was taken to be 9·10 millipoises (by intrapolation in *International Critical Tables*). The sp.gr. of collodion was taken to be 1·66 (Petitpas, 1948), and the volume of solvent in the membrane was calculated using this figure, the dry matter content and the linear dimensions of the membranes. These results are set out in Table 2.

Apparatus

The electrokinetic ultrafiltration was done in strips of wet collodion membrane surrounded by an electrically insulating liquid to prevent overheating and evaporation. It was necessary that this liquid should not swell or dissolve the collodion. Chlorobenzene (cf. Cremer & Tiselius, 1950) was satisfactory in early experiments, in which a strip of membrane was pushed through a U-tube nearly filled with chlorobenzene so that the ends projected above the surface of the chlorobenzene in each limb of the tube into the anolyte and catholyte respectively, which were small volumes of 0·2N aqueous acetic acid overlying the chlorobenzene. However, this arrangement left some uncertainty as to the point at which the anolyte entered the strip and also required inconveniently large volumes of anolyte when it was wished to study material only available in small quantity. The arrangement shown in Fig. 1 was finally adopted, using liquid paraffin B.P. that had been equilibrated with a large volume of 0·2N aqueous acetic acid as the insulating liquid. The soft sheet rubber used for the gaskets of the anolyte chamber swelled somewhat in contact with the paraffin and this ensured the absence of leaks; the anolyte was thus able to enter the strip only through its end surface and the anolyte chamber was conveniently small for work with mg. quantities. New gaskets were used for each run.

Procedure

A strip (1·3 × 8·0 cm.) was cut from the membrane and blotted with filter paper to remove adhering liquid. Working quickly to avoid drying the strip, a hole was punched in it near the cathode end and the anode end was mounted in the anolyte chamber which was firmly gripped with a 'bulldog' clip. The strip, held in this way, was then mounted in the test tube so that the hole was hooked by the cathode as shown in Fig. 1. The test tube was then filled to about the level of the top of the cathode with 0·2N acetic acid and then to the level of the junction of the strip and anolyte chamber with paraffin. The sample for analysis (usually 0·5–1·0 mg. polysaccharide) in *ca.* 0·1 ml. of suitable solution was placed in the anolyte chamber with a micropipette. The anode was then lowered centrally into the anolyte nearly to touch the strip, and a p.d. of 250 V was applied across the electrodes. Currents, which did not vary greatly with the porosity of the membranes, were usually in the range 1·0–2·5 mA. When the anolyte had nearly entered the strip (the time taken for this, determined by the rate of electroendosmosis, varied greatly with the porosity of the membrane used—see below), the anode was removed and cleaned and the anode chamber rinsed out with 0·2N acetic acid; the anode was then

Table 1. *Mixtures used for preparing collodion membranes*

Membrane	Necol solution (ml.)	Ethanol (ml.)	Ether (ml.)	Amyl alcohol (ml.)	Acetone (ml.)	Ethylene glycol monoethyl ether (Cellosolve) (ml.)
I	64	14·2	152	24·8	64	7·1
II and III	95	30	150	25	40	3
IV	95	30	150	25	30	3
V	355 ml. of a mixture of equal parts by wt. of above			—	—	—
VI	The same, allowed to evaporate to a more compact gel			—	—	—

Table 2. *Characterization of collodion membranes*

For experimental details and methods of calculation see text.

Membrane	Thickness (mm.)	Dry matter content (%)	Vol. fraction occupied by solvent (V_L)	Rate of passage of solvent (ml./hr.)	Applied pressure (cm. Hg)	'Average pore radius' (mμ.)	S*
I	0·53	17·8	0·884	333	19·8	552	y
II	1·07	16·9	0·891	1·99	19·5	61	—
III	0·96	20·6	0·865	0·42	19·3	27	$20{\cdot}4y$
IV	0·79	25·9	0·825	0·19	19·3	16·7	$33{\cdot}1y$
V	0·76	25·0	0·832	0·059	19·5	9·1	$60{\cdot}8y$
VI	0·76	23·8	0·840	0·048	19·3	8·2	$67{\cdot}3y$

* See Discussion.

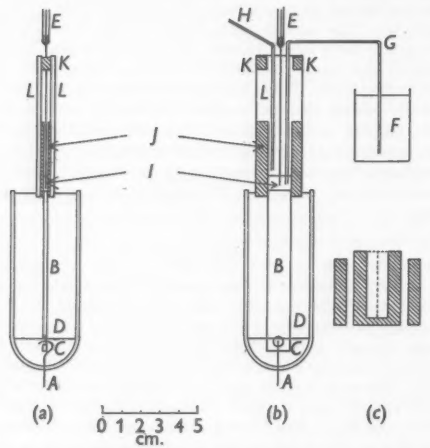

Fig. 1. Apparatus for electrokinetic ultrafiltration in strip of collodion membrane: (*a*) strip seen from side; (*b*) strip seen from front; (*c*) details of sheet-rubber gaskets. The two side gaskets are cut from the piece of rubber used for one of the two main gaskets as indicated by dotted lines. *A*, platinum-wire cathode sealed through bottom of glass tube; *B*, collodion strip; *C*, catholyte (0·2 N acetic acid); *D*, liquid paraffin (see text); *E*, platinum-wire anode, sealed into glass tube containing mercury; *F*, reservoir of 0·2 N acetic acid; *G*, capillary siphon for perfusing anode chamber in long runs; *H*, capillary connected to filter pump for sucking perfusion liquid from anode chamber; *I*, anolyte; *J*, sheet-rubber gaskets surrounding anolyte-membrane junction; *K*, rubber spacing pieces; *L*, glass microscope slides. The microscope slides are pressed together by a 'bulldog' clip (not shown), causing them to grip the end of the collodion strip and to seal the anolyte chamber.

replaced and the voltage reapplied. When electroendosmosis was slow and the run long the anode chamber was perfused with 0·2 N acetic acid admitted slowly by the capillary siphon (Fig. 1) from an adjacent beaker and removed by continuous suction. This prevented accumulation of anodic reaction products; however, perfusion was not necessary with shorter runs, during which the anolyte was replenished at intervals by hand. The time for running in the sample was not allowed to exceed one-sixth of the total running time.

Stains for polysaccharide zones and markers of electroendosmosis. At the end of the run the strip was removed and blotted free of paraffin. The polysaccharides studied in the present work were revealed by spraying the strip lightly on both sides with 0·1 N-I_2 in 0·15 M aqueous KI. After a few minutes coloration of the zones reached its maximum and measured sketches or colour photographs were prepared. The iodine stain of the polysaccharides of lowest DP was evanescent but that of most of the materials studied was surprisingly permanent during 1–2 yr., although the strips twisted and shrank badly as they dried. The zones were sometimes rather curved in form, having migrated more slowly at the edges than at the centre of the strip (cf. Fig. 4). This could be due to uneven distribution of electric field and temperature across the strip and possible drying at the edges. All measurements of zones were made in the midline of the strip.

When it was wished to measure electroendosmosis, 10% (w/v) of fructose was incorporated in the solution taken for analysis. It had been shown (see below) that fructose is not adsorbed or retained by the collodion. At the end of the run the strip was divided along its midline; half was stained with iodine and the other half was divided along its length into about 20 segments, each of which was placed in 1 ml. of water in a test tube for 1 hr. At the end of this time the 'rapid furfural' test (Cole, 1933) was done on each of the eluates, thus revealing the position of the fructose zone. Several of the usual colour reactions for sugars by direct spraying of the strip were tried, but did not seem to work well on collodion; the most encouraging was spraying with sat. ethanolic urea oxalate (suggested by Dr P. C. Arni; cf. Hirst, McGilvray & Percival, 1950), but the subsequent heating at 105° which revealed the fructose as a dark zone shrank and distorted the strip. *N*-2:4-Dinitrophenylethanolamine, which we have found useful as a coloured neutral marker for electroendosmosis in other electrophoretic work (e.g. Mould & Synge, 1954), was useless as it was retained by the collodion, forming an immobile zone at the anode end of the strip.

Polysaccharides

To avoid trouble from retrogradation all solutions were prepared immediately before each experiment.

Amylose. Preparations by the alumina–thymol procedure (Hobson, Pirt, Whelan & Peat, 1951) from potato starch were given to us by Dr P. N. Hobson and by Dr W. J. Whelan. For electrokinetic ultrafiltration runs, samples (2 mg.) were dissolved with heating in 0·1 ml. 0·04 N-NaOH. On cooling, 0·1 ml. 0·2 N acetic acid was added and 0·1 ml. of the resulting solution was used for the run. Some runs were done with undried thymol complex brought into solution by Dr Hobson as follows. Wet suspension (1 g., calculated to contain 50 mg. amylose) was heated for 5 min. at 100° with 10 ml. of solution 0·02 N in sodium acetate and 0·18 N in acetic acid. This gave an opalescent solution, which on cooling became cloudy. This was centrifuged and the clear supernatant, which gave a strong blue stain with iodine, was used for the experiments.

Partially hydrolysed amylose. Amylose (80 mg.) was dissolved in a minimum of 10 N-HCl and kept for 10 min. at room temperature. The mixture was then evaporated to dryness *in vacuo* not allowing the temperature to exceed 30° or the time of evaporation to exceed 15 min. Water was added, and evaporation repeated 2–3 times to decrease the concentration of HCl. The residue was dissolved in 4 ml. water and neutralized to pH 4 with sodium acetate. This procedure gave a representative mixture of dextrins suitable for many tests on methods. The mixture stained purple with iodine. If a mixture having a rather narrower range of DP was desired, the hydrolysis was effected with salivary α-amylase. The degree of hydrolysis was controlled by observing the change in 'blue value' (B.V.) of the polysaccharide when it is stained with iodine under the conditions specified by Bourne, Haworth, Macey & Peat (1948). Human saliva was centrifuged to remove mucin and the clear supernatant used as the enzyme source. The α-amylase activity was not standardized. Potato amylose (250 mg.)

was wetted with ethanol and dispersed in 20 ml. N-NaOH at 100° for 5 min., neutralized with N-HCl to pH 5, 50 ml. 0·4M sodium acetate buffer (pH 4·7) added and the solution made up to 250 ml. at pH 4·8. The salivary α-amylase preparation (0·5 ml.) was added. Samples (1 ml.) were taken during hydrolysis and made up to 100 ml. with I_2 (2 mg./100 ml.) and KI (20 mg./100 ml.) for determination of B.V. For extended hydrolysis a further 0·5 ml. of saliva preparation was added. Enzymic hydrolysis was terminated by heating to 100°; the denatured protein was then removed by centrifuging. A blue-purple coloration with iodine corresponded to B.V. 0·56.

Phosphorylase-synthesized linear dextrins. These were preparations provided by Dr W. J. Whelan, made from pure amylohexaose or amyloheptaose and having average DP as specified (Whelan & Bailey, 1954). However, one preparation studied, having average DP 75·5 before dialysis, had been made from a heterogeneous primer mixture. This primer mixture was obtained by acid hydrolysis of potato amylose (1%, w/v) in 0·25N-H_2SO_4 at 100° for 15 min. After cooling and neutralizing (6N-NaOH) the average DP was measured by determination of reducing power (Whelan, Bailey & Roberts, 1953) and found to be 6·0. In using this material for amylose synthesis, it was assumed that all molecules present acted as primers. This assumption is incorrect in so far as any glucose and maltose present are devoid of priming activity. In consequence the true average DP must have been greater than 75. Samples were dissolved as described above for amylose. As noted by others (cf. Whistler & Johnson, 1948), samples having DP above 50 were considerably less easy to dissolve in the alkali than potato amylose. However, heating never exceeded 10 min. at 100° and experiments were done with whatever material had dissolved under these conditions.

Electrophoretic fractions of partially hydrolysed amylose. These were worked up after an electrophoretic run as described by Mould & Synge (1954). For electrokinetic ultrafiltration experiments they were dissolved as described above for amylose.

Amylopectin. A sample of potato amylopectin (B.V., 0·164) prepared by the method of Hobson, Pirt, Whelan & Peat (1951) was provided by Dr Whelan. It dissolved readily, as above, for the runs without heating.

Potato amylopectin β-limit dextrins. These were freeze-dried dextrins prepared by the method described by Hobson, Whelan & Peat (1950) from a thymol amylopectin and from a thymol amylopectin which had been purified by adsorption of the last traces of amylose on a cellulose column. They dissolved readily without heating.

Products of action of 'debranching' enzyme. Hobson, Whelan & Peat (1951) have described the isolation of an enzyme, 'R-enzyme', from broad beans and potatoes which effects the scission of the branch linkages in amylopectin and amylopectin β-dextrin. The isolation, on a large scale, of the products of action of this enzyme ('R-dextrins') which have been used in this work will be described in detail in a forthcoming paper (Peat, S., Hobson, P. N., Whelan, W. J. & Thomas, G. J., to be published), but relevant details are given here. A thymol-amylopectin from potato starch was further purified by fractionation with methanol (Hobson, Pirt, Whelan & Peat, 1951) and from part of the product a β-dextrin was prepared as described by Hobson *et al.* (1950). The amylopectin and β-dextrin were treated with bean R-enzyme on a large scale and the products separated into fractions diffusible and indiffusible through cellophan. The indiffusible fractions were used here. In each case the total recovery was 90% of the amount of polysaccharide started with and the indiffusible fraction in the case of the amylopectin R-dextrin was 84·0% of the starting material and in the case of the β-dextrin R-dextrin 63·5%. The B.V.'s of the dextrins were 0·320 and 0·265 respectively. The R-dextrin from the β-dextrin of waxy maize starch was prepared in a similar manner, the indiffusible portion representing 49·0% of the initial β-dextrin, and the B.V. of the R-dextrin being 0·285. The materials dissolved readily as above in the cold or with gentle warming.

Amylose β-dextrin. The dextrin was prepared by Dr Gwen J. Thomas as follows. Potato amylose (B.V., 1·40; 2·922 g. dry wt.) was placed in a 2 l. standard flask and moistened with ethanol (5 ml.). Water (500 ml.) and 6N-NaOH (14 ml.) were added and the flask was heated in a boiling-water bath for 5 min. The flask was cooled, the contents were diluted to 1800 ml. with water and neutralized (6N-H_2SO_4). 0·2M Sodium acetate buffer (pH 4·8; 50 ml.) and crystalline sweet-potato β-amylase suspension (5000 units; Hobson *et al.* 1950) were added and the digest was diluted to 2 l. During incubation at 35° the course of reaction was followed by measurement of intensity of iodine stain. After 26 hr., when the reaction appeared to be complete and the intensity of stain had fallen to the usual level, the digest was heated in a boiling-water bath for 10 min. and the cooled solution dialysed against running tap water for 2 days to remove salts and maltose. The solution was concentrated under reduced pressure to 100 ml. and freeze-dried. The yield of dextrin was 0·953 g. having a B.V of 1·23. The dextrin dissolved incompletely after heating for 5 min. at 100° in the NaOH. However, the supernatant after acidification gave a deep-blue stain with iodine.

Determination of adsorption equilibria in collodion membranes

Equilibration by diffusion. (i) *Fructose.* Square portions of a collodion membrane similar to VI (Tables 1 and 2), blotted with filter paper and having wet weight 0·17–0·23 g. were placed in tared stoppered weighing bottles and weighed. Approximately 0·8 ml. of solutions of D-fructose in 0·2N acetic acid ranging from 0·1 to 10% (w/v) were then added to each weighing bottle; the bottles were again weighed. After equilibrating for 24 hr. at room temperature, weighed portions of the supernatants (0·2–0·3 g.) were transferred to tared tubes, evaporated to dryness *in vacuo* over H_2SO_4 at room temperature and the dried residues were weighed. Similar portions of the original fructose solutions were analysed gravimetrically in the same way. Eight different concentrations of fructose were used and a control portion of membrane was kept in 0·2N acetic acid only. The control supernatant contained 0·27 mg. dry matter/g. which had passed into solution from the collodion. On deducting this from the concentrations of dry matter found in the experimental supernatants, we calculated the total remaining dissolved dry matter, assuming it to be evenly distributed between the supernatant liquid and the liquid inside the pores of the membrane (cf. Table 2). We thus obtained weights of unadsorbed fructose not significantly different from those taken initially for each experiment (Table 3). This proves that fructose at the concentration of 10% (w/v) and slightly less, as used in the marker experiments, is not significantly adsorbed on the collodion of the membranes

Table 3. *Equilibrium adsorption of fructose on collodion membrane*

For experimental details and calculations see text.

Approx. final fructose concentration (% w/v)	Fructose taken (mg.)	Fructose found in liquid (mg.)	Difference (adsorbed fructose) (mg.)
10	95·10	96·10	−1·00
5	45·70	45·80	−0·10
2	18·45	18·38	0·07
1	8·94	9·30	−0·36
0·5	4·95	4·78	0·17
0·2	2·19	2·11	0·08
0·1	1·25	1·03	0·22

and is therefore a reliable marker for electroendosmotic flow in the membranes.

(ii) *Linear dextrins.* Wet collodion membrane (1 g.) was cut into small squares (approx. 3 × 3 mm.) and added to 10 ml. of a solution of polysaccharide in 0·2 N acetic acid. The flask containing this was shaken continuously for 24 hr. at room temperature. Then 0·5 ml. of supernatant was removed, diluted to 25 ml. and this solution was made 0·001 M in respect of KI. One ml. 0·001 N-I_2 in 0·001 M-KI was added and after 7 min. the light absorption at the absorption peak was measured in a Beckman DU spectrophotometer. The polysaccharide concentration was found by intrapolation in a calibration curve made using dilutions of the original polysaccharide solution. Shaking of the flask was continued, samples being taken at 48 and 96 hr., but it was found that the increase in adsorption after 24 hr. was not significant.

Equilibration of polysaccharides by mechanical ultrafiltration. A circular section of collodion membrane (effective diam. 4·6 cm.) was placed between rubber gaskets above the perforated plate of a Seitz filter. The filter was filled with 25 ml. of polysaccharide solution in 0·2 N acetic acid and a positive pressure of N_2 (1 kg./cm.^{2}) was applied. Fractions of filtrate (0·5 ml.) were collected with a small fraction collector and the polysaccharide concentration in each was determined spectrophotometrically as above. The rate of flow of solution through the membrane was also measured. The course of an experiment using electrophoretic fractions of an amylose hydrolysate is shown in Fig. 2. The shape of the curves and the fact that the polysaccharide content of the filtrate approaches that of the original solution and does not decrease shows that adsorption proceeds until equilibrium is reached and that there is no sieving action or blocking of the pores (Elford, 1933). The different adsorption of the different polysaccharides was calculated from the retention volumes found by integrating the curves, and was expressed as mg. polysaccharide/mg. dry collodion for the given polysaccharide concentration.

RESULTS

Rate of electroendosmosis

The electroendosmotic permeability D_I, (i.e. the liquid volume (ml.) electroendosmotically transported in 1 sec. by a current of 1 A, across 1 cm.^{2} cross-section of membrane) measured in the course of the experiments for the different membranes is plotted in Fig. 3 against the pore radius of the membranes (cf. Table 6). Absolute rates of electroendosmosis ranged from 0·2 cm./hr. for the finest membranes to 6 cm./hr. for the coarsest.

R_F values of phosphorylase-synthesized polysaccharides (homogeneous primers)

Values for these were determined on the various membranes by developing until the fructose zone had nearly approached the cathode end of the strip. More accurate estimates of R_F for the slower-moving bands could be obtained by running for longer and measuring the rate relative to some zone other than fructose; incorporation of such data does not materially alter the results. Breadths of bands and R_F values are set out in Table 4. Since all the zones appeared reasonably symmetrical, the R_F value of the centre of the band has been assigned to the polysaccharide component having the average DP for each fraction as determined from the phosphate-liberation data. Where bands resolved well from one another, several R_F values could be determined in one strip. Fig. 4 is a sketch of a developed strip

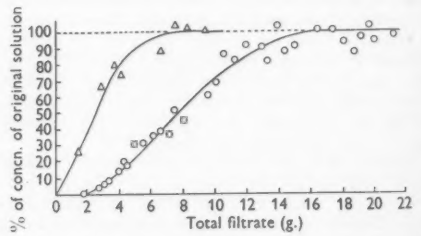

Fig. 2. Retention of electrophoretically fractionated linear dextrins dissolved in 0·2 N acetic acid during flow through collodion membrane IV: △, red-staining fraction (0·548 mg./ml.; rate of flow, 0·13 ml. hr.$^{-1}$ cm.$^{-2}$); ○, blue-staining fraction (0·387 mg./ml.; rate of flow, 0·29 ml. hr.$^{-1}$ cm.$^{-2}$). For further details see text.

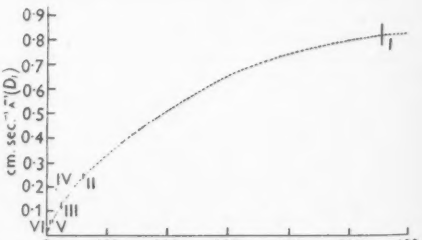

Fig. 3. Electroendosmotic permeability of the collodion membranes in relation to average pore radius (see text and Table 2). The vertical lines give the extreme range of values of D_I observed with each membrane.

Table 4. *Ranges and mean values for R_F of linear dextrins in different collodion membranes*

The values in the extreme right-hand column are arithmetical means of the two adjoining values.

Average DP of polysaccharide preparation	No. of expts.	R_F: Extreme values, Leading edge	R_F: Extreme values, Trailing edge	R_F: Mean values, Leading edge	R_F: Mean values, Trailing edge	Centre of zone
Membrane I						
47·5	2	—	—	—	—	1
103	2	1	0·53	1	0·58	(0·79)
205	1	0·73	0·45	—	—	0·59
250	1	0·66	0·21	—	—	0·43
Membrane III						
34	3	0·79	0·61	0·79	0·61	0·70
77	3	0·50	0·21	0·44	0·28	0·36
103	1	0·46	0·21	—	—	0·33
160	2	0·16	0·08	0·15	0·08	0·115
Membrane IV						
34	2	0·93	0·55	0·85	0·60	0·72
103	3	0·37	0·12	0·35	0·14	0·245
160	1	—	—	—	—	(0·1)
Membrane V						
34	4	0·56	0·38	0·54	0·40	0·47
47·5	1	0·50	0·31	—	—	0·405
77	4	0·25	0·10	0·22	0·11	0·165
103	1	0·18	0·09	—	—	0·135
160	1	—	—	—	—	Zero
Membrane VI						
34	4	0·54	0·29	0·52	0·32	0·42
77	3	0·22	0·09	0·21	0·10	0·155
103	1	—	—	—	—	Zero
160	1	—	—	—	—	Zero
205	1	—	—	—	—	Zero
250	1	—	—	—	—	Zero

showing three such resolved bands. The polysaccharides with average DP 34 and 47·5 showed colour play within the zones, the leading parts staining pinker with iodine and the trailing parts bluer. The other polysaccharides all gave a uniform blue reaction throughout the zones.

Adsorption results for linear polysaccharides of known DP: calculation of expected R_F values

Adsorption results with membrane IV for a 'blue' electrophoretic fraction of hydrolysed amylose having DP approx. 40–90 (see below; Mould & Synge, 1954) were obtained by the diffusion and ultrafiltration procedures each at two different concentrations and are shown in Fig. 5. The results by the two different methods are seen to lie reasonably close to the same postulated isotherm. Similar isotherms were obtained for a 'red' electrophoretic fraction (DP *ca.* 25–40; see below) with membranes IV and VI and with an enzyme-synthesized preparation (average DP 77) with membrane VI. Points on these isotherms (as shown in Table 5) were used for calculating the R_F values expected, assuming that adsorption is the only factor retarding the polysaccharides. The calculation was done as follows. The formula of Consden, Gordon & Martin (1944) gives

$$R_F = \frac{A_L}{A_L + \alpha A_S},$$

where A_L = cross-section of moving phase, A_S = cross-section of stationary liquid phase, α = partition coefficient $= \frac{[\text{solute}]_{\text{stationary phase}}}{[\text{solute}]_{\text{moving phase}}}$. For adsorption chromatography and considering volume rather than cross-section, we may rewrite the equation thus:

$$R_F = \frac{V_L}{V_L + aM_S}, \qquad (1)$$

where V_L = volume of moving phase in unit volume of chromatogram (see Table 2), M_S = mass of adsorbent in unit volume of chromatogram (see Table 2), a = slope of adsorption isotherm $= \frac{\text{mass of solute adsorbed/unit mass of adsorbent}}{\text{concentration of solute in moving phase}}$.

The R_F values calculated using equation (1) from direct determinations of adsorption are compared in Table 5 with those found in electrokinetic experiments (Table 4 and below under 'Electrophoretic fractions'). It is seen that the former, while exhibiting the same trends, are consistently lower than the latter.

Enzyme-synthesized polysaccharide from heterogeneous primer

This material, which gave a clear blue stain with iodine, scarcely entered membranes (similar to V and VI) that were freely penetrated by material of DP 77 made from homogeneous primer. On membrane I it had R_F 0·57–0·80, but the trailing edge of the zone was sharp while the leading edge was diffuse. This suggests a DP range 110–200, with a predominance of the higher polymers.

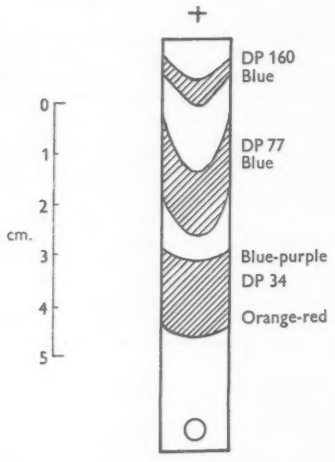

Fig. 4. Resolution of phosphorylase-synthesized linear dextrins (average DP of preparations 34, 77 and 160) on membrane III. Portions (1 mg.) of each preparation were heated at 100° in 0·2 ml. 0·04 N-NaOH. After cooling, 0·2 ml. 0·2 N acetic acid was added. This mixture was run into the strip for 30 min. and the run continued with 0·2 N acetic acid as anolyte for a further 450 min. The DP 160 preparation dissolved poorly, the corresponding band being weaker than the other two.

Amylose hydrolysates

These were much used for orienting experiments but were not subsequently studied in detail. HCl-hydrolysed amylose gave broad continuous zones and, in the finer-pored membranes, showed a brilliant series of changes of iodine colour in the parts of the zone corresponding with DP below about 40, the blue colour being preceded by a narrow purple region, then pink, then orange; the orange colour was not permanent, but disappeared rather rapidly as iodine evaporated from the strip. Partial hydrolysates made with saliva showed the same play of colours, but their zones did not cover such a wide range of R_F values. In each case increasing the extent of hydrolysis increased the R_F value for the trailing edge of the zone and increased the concentration in the red- and orange-staining part of the zone.

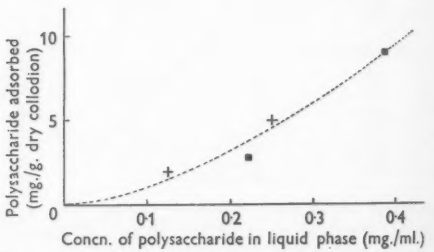

Fig. 5. Adsorption isotherm for electrophoretically fractionated dextrin (DP 40–90) on membrane IV. +, Equilibration by diffusion; ■, equilibration by mechanical ultrafiltration. For details, see Experimental section.

Table 5. *Adsorption data for polysaccharide preparations on collodion membranes related to R_F values*

For details see text.

Membrane				Adsorption data			R_F	
No.	V_L	M_S (g./ml.)	Polysaccharide	Polysaccharide (mg.) adsorbed/g. dry collodion	Concn. polysaccharide (mg./ml.)	a (ml./g.)	Calc. from adsorption data	Found in electrokinetic experiments
IV	0·825	0·29	Electrophoretic 'blue'	3·8	0·225	16·9	0·14	0·48*
IV	0·825	0·29	Electrophoretic 'red'	3·8	0·548	6·9	0·29	0·73*
VI	0·840	0·265	Enzyme-synthesized (av. DP 77)	4·0	0·125	32·0	0·09	0·155
VI	0·840	0·265	Electrophoretic 'red'	4·5	0·400	11·3	0·22	0·43

* By intrapolation—see Table 4 and Fig. 7.

Electrophoretic fractions

'Orange' fractions could not conveniently be studied, owing to the low sensitivity and impermanence of the iodine staining. Experiments were done with 'red' and 'blue' fractions from a salivary-amylase hydrolysate of potato amylose (B.V. 0·56). In membrane V the 'red' material had R_F 0·40–0·58 and gave a uniform pink coloration. From Fig. 7 this would indicate DP range 25–40. The front of the zone of 'blue' material, which showed a slight purplish tinge, had R_F 0·40, and the zone, the rest of which was blue, extended back to R_F 0·07, corresponding to DP approx. 120. A mixture of the two fractions gave a single zone with no gap in the region R_F 0·40.

A second specimen of 'red' material (amylose hydrolysis to B.V. 0·42) tested on membrane VI had R_F 0·37–0·49, indicating DP range 27–42, while pooled 'blue' fractions from a number of salivary hydrolysates had R_F 0·06–0·38 suggesting DP range 40–90. Appearances in this experiment were the same as in the previous one, and a mixture of the 'red' and 'blue' fractions also behaved in the same way.

The significance of these observations is discussed in the following paper (Mould & Synge, 1954).

Amylose

This did not enter the finer membranes, forming a skin of blue-staining material on the anode end, which could be removed by gentle scraping. In the coarse membrane I an intense blue zone extended from the origin to R_F 0·06, preceded by pale-blue coloration as far as R_F 0·4. This suggests that the substances of lowest molecular weight present had DP *ca.* 250 and that most of the preparation was of substantially higher DP. Results were the same with dried material brought into solution with alkali and the undried thymol complex brought into solution without alkali. A second preparation of amylose by the alumina–thymol procedure gave rather more material with R_F 0·06–0·4.

Amylose β-dextrin

With membrane I this gave a picture not different from those obtained with amylose above.

Amylopectin

This did not enter the finer membranes. In I there was an intense zone from the origin to R_F 0·05, a weaker region extending to R_F 0·2 and traces of staining up to R_F 0·4. All the staining had a uniform purple colour.

Amylopectin β-limit dextrin

This was not distinguishable from amylopectin in its distribution on the strips, although it gave a pinker staining reaction.

Debranched preparations

The preparations both from potato amylopectin and from its limit dextrin were sharply separated, on the coarse membrane I, into a fast-running zone (R_F 0·8–1·0) and a slow-running zone (R_F 0–0·04). With debranched amylopectin the fast zone stained blue-purple and the slow zone purple. With the debranched limit dextrin the fast zone stained blue-purple but had a pink leading edge, while the slow zone stained pink-purple. The product from waxy maize starch gave a similar picture.

To characterize more closely the fast-moving fractions the debranched products were studied on membrane IV. Again in each case there was purple-pink staining material which scarcely entered the membrane. With the debranched potato amylopectin, the material entering the membrane gave a zone having R_F 0·14–0·56, staining blue, the front part more intense and having a slight purple tinge. With the product from the corresponding limit dextrin the faster zone had R_F 0·26–0·75 and had the characteristic orange-pink colour play towards its leading edge. The debranched limit dextrin from waxy maize starch likewise showed much material unable to enter the membrane and a zone R_F 0·42–0·9 with characteristic colour play and a concentration of orange-staining material at its leading edge.

These results suggest that, if the faster moving products of the enzyme action are linear amylodextrins, they lie, for debranched potato amylopectin in the range DP 60–140 and for the corresponding debranched limit dextrin in the range DP 25–110 (Fig. 7). The products from the debranched limit dextrin from waxy maize starch have an upper DP limit *ca.* 75, but the lower limit cannot easily be determined owing to the poor staining with iodine. In each case there seems to be a preponderance of material towards the lower end of the DP range.

DISCUSSION

Physicochemical mechanisms determining the separations

As explained in the introduction to this paper, it was hoped, by the procedure adopted, to make use of molecular-sieve effects in so far as they could help the desired fractionations and at the same time to prevent them from interfering with any separations due primarily to adsorption. We feel that this object has been achieved; however, it remains to ascertain the parts played by the two effects. The fact that with the enzyme-synthesized polysaccharides compact zones with definite R_F values could be obtained indicates that any adsorption was according to approximately linear isotherms and that any retardation by molecular-sieve action was

for each solute a constant proportion of the electroendosmotic flow velocity irrespective of solute concentration.

The results on adsorption of linear dextrins obtained either by diffusion or by determining retention volumes during mechanical ultrafiltration across the membrane gave mutually consistent values for the adsorptions. This agreement, the form of the 'frontal analysis' curves from the mechanical ultrafiltrations (Fig. 2) and the fact that the effluent concentration rises to that of the original solution strongly suggests that with these dextrins that can penetrate the membranes only simple adsorption on the collodion surfaces need be postulated to explain the retardation. In fact, an explanation is demanded as to why the apparent adsorption should be less under electrokinetic ultrafiltration conditions than otherwise.

It should be noted that in the finer membranes the electrokinetic flow was 10–100 times greater than the mechanical flow, and would give rise to a correspondingly greater shear. Further, under the electrokinetic conditions the shear gradient is probably steeper near the collodion surfaces than during mechanical flow, giving rise to greater absolute shears at the surfaces. The work of Silberberg & Kuhn (1952) on disturbance of equilibria by shear in liquid–liquid systems is relevant in this connexion, and one could well imagine a similar effect of shear in modifying adsorption equilibria at solid surfaces. However, the adsorption found during free diffusion and mechanical ultrafiltration may well have been increased by irreversible adsorption or retrogradation, since it proved impossible by continuous washing of the collodion with 0·2N acetic acid to remove completely iodine-staining material after the experiments. Such irreversible phenomena cannot have occurred in the electrokinetic work, since the migrating zones left no tail of iodine-staining material. The high shear perhaps also acted to check retrogradation. This problem requires further study with materials which, unlike these linear dextrins, are in more stable equilibrium in the dissolved state.

As to the actual rates of electroendosmotic flow of 0·2N acetic acid in the membrane, it is seen (Fig. 3) that this varies with the pore radius of the membranes, increasing approximately linearly with pore radius and then falling off, presumably to approach the constant velocity independent of pore radius required by the Helmholtz–Smoluchowski equation. The similarity of the whole curve to that obtained from the extensive data of Manegold & Solf (1931) indicates that the general electroendosmotic behaviour of the membrane in a direction parallel to its surface is the same as in the more usually investigated transverse direction. The theoretical treatment of Schmid (1951), applied by him to the data of Manegold & Solf, is probably only applicable up to a pore radius of 10 mμ. (i.e. our membranes V, VI), the thickness of the double layer being of this order for 0·2N acetic acid. The hydrostatic permeability D_H, i.e. the volume (cm.^{3}) of 0·2N acetic acid flowing through 1 cm.^{2} of membrane/sec. under a pressure of 1 cm. water, can only be measured for flow across the membrane. D_H cannot therefore be strictly compared with D_I without making an arbitrary assumption as to the probable effect of the difference in thickness of the membranes under the two experimental conditions. The values of D_I, D_H, D_I/D_H are shown in Table 6. Assuming a constant factor for the thickness effect it can be seen, however, that D_I/D_H is approximately constant for the smallest pored membranes and then markedly decreases with increasing pore radius, in agreement with the data of Manegold & Solf and the theoretical treatment of Schmid. O. N. Grigorov (quoted by Zhukov, 1943) obtained similar results.

Further tests were applied to see whether surface adsorption on the membrane structure could give an adequate explanation of the R_F values observed. As all the membranes were made from the same commercial collodion, the amount of a given polysaccharide adsorbed at given concentration should be directly proportional to the adsorbing surface area in unit volume of membrane irrespective of the porosity. Although the 'pore radii' calculated as above cannot be simply related to the complicated actual structure of the collodion membrane, if it is assumed that the pore structure of each membrane has a similar geometry, differing only in scale, then these dimensions will be related linearly to the 'pore radius' (cf. Sullivan & Hertel, 1942). Surface area of the membrane structure/unit volume of membrane thus varies inversely with the first power of

Table 6. *Comparison of electroendosmotic and hydrostatic permeabilities of membranes*

For explanation see text.

Membrane	Pore radius (mμ.)	D_I (cm.sec.$^{-1}$A^{-1})	D_H ($\times 10^6$)	D_I/D_H ($\times 10^{-4}$)
I	552	0·836	685	0·122
II	61	0·240	4·1	5·85
III	27	0·116	0·903	12·8
IV	16·7	0·185	0·395	46·7
V	9·1	0·055	0·124	44·4
VI	8·2	0·049	0·102	48·0

the 'pore radius'. (Altering the linear dimensions of a given structure by the factor x changes its surface area by the factor x^2 and its volume by x^3. The surface area now present in the original volume is therefore x^2/x^3, i.e. $1/x$.)

Equation (1) may be rewritten substituting bS for aM_s where b is the slope of the adsorption isotherm redefined in terms of area as

$$\frac{\text{mass solute adsorbed/unit area of adsorbing surface}}{\text{concentration of solute in moving phase}},$$

and S is area of adsorbing surface/unit vol. of membrane. Rearranging,

$$b = \frac{V_L}{S}\left(\frac{1}{R_F} - 1\right). \qquad (2)$$

For membrane I an arbitrary value y is assigned to S. The values of S for the other membranes then vary inversely as pore radius and are shown in Table 2. Substituting these and the appropriate values for V_L and R_F in (2), values of b in terms of y can be calculated for the data in the last column of Table 4. Values of $\log_{10} by$ for each polysaccharide and membrane are plotted against DP in Fig. 6. The values for a given polysaccharide are fairly independent of membrane, confirming that surface adsorption can explain the phenomena observed. Furthermore, all the points lie fairly close to a single straight line, showing that the adsorption affinity increases with DP according to Traube's Rule. However, the three points for membrane I might be regarded as departing from this line and a second line has been drawn for them only. This deviation may reflect a change in structure of the membranes between I and III, which is in keeping with the idea of a critical region postulated for II and intermediate solvent mixtures between those for I and II (see above).

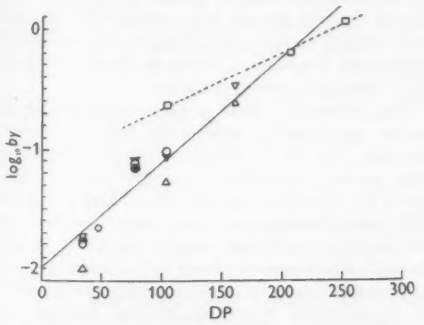

Fig. 6. Relation of adsorption on internal surfaces of collodion membranes with DP of the polysaccharides. The full line relates to the data for all membranes (16 points), the dotted line to the data for membrane I only. For details of calculation see text. Membranes represented as follows: □, I; ▽, III; △, IV; ○, V; ●, VI.

The Traube's Rule relationship illustrated in Fig. 6 holds, however, only for those substances which have finite R_F values. R_F fell to zero with increase of DP sooner than could be expected from Traube's Rule and molecular-sieve effects must be postulated to explain this. They can be the only explanation where solute was actually scraped off the anode end of the membrane. We were rather surprised that these molecular-sieve effects set in over such a comparatively narrow range of DP—we had expected they would manifest themselves more gradually, and Barrer (1949) held the same opinion for ultrafiltration membranes made from organic materials. This seems to be a further testimony to the uniformity of pore size of this type of collodion membrane.

It is interesting that the converse adsorption of dissolved collodion on to starch grains has been used by Brooks & Badger (1950) for the chromatographic fractionation of collodion.

Range of DP in the enzyme-synthesized polysaccharide preparations

By plotting the data of Table 4 curves can be constructed showing variation of R_F with DP for the different membranes. The breadth of the zones can then be used to define the range of DP represented in each polysaccharide preparation. This has been done in Fig. 7. The DP ranges found with the different membranes are shown synoptically in Table 7. Although some of the experiments were rather crude, agreement is reasonable where experiments were repeated several times with different membranes.

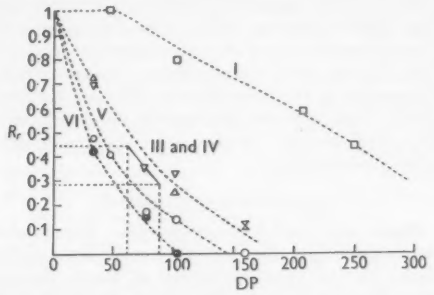

Fig. 7. Relation of R_F to DP for the polysaccharides in the different membranes. The points are the data of Table 4 for zone centres. The slope of the appropriate curve in the neighbourhood of each experimental point has been used for converting the R_F values of the zone edges (Table 4) to the DP ranges given in Table 7. Membranes represented as follows: □, I; ▽, III; △, IV; ○, V; ●, VI. Graphical conversion of the data for the preparation having average DP 77 on membrane III is illustrated as an example.

Table 7. *Ranges of DP judged to include 95 % of molecules in enzyme-synthesized polysaccharide preparations*

See Fig. 7 and text.

	DP (glucose residues/molecule)						
	Average DP of preparation						
	34	47·5	77	103	160	205	250
Membrane I	—	—	—	—	—	140–250	170–320
Membrane III	21–46	—	62–92	75–130	150–170	—	—
Membrane IV	20–49	—	—	75–128	—	—	—
Membrane V	27–42	35–62	63–90	89–115	—	—	—
Membrane VI	22–46	—	67–85	—	—	—	—
Extreme range for all membranes used	20–49	35–62	62–92	75–130	150–170	140–250	170–320
Mean range for all membranes used	23–46	35–62	64–89	80–124	150–170	140–250	170–320
Spread of DP in mean range	23	27	25	44	20	110	150

The zones will be wider than ideally on account of diffusion, heteroporosity, etc., and also because the material was applied as a zone of finite width. However, the finite sensitivity of the iodine-staining reaction works in the opposite direction. We do not think it unreasonable to assume that 95 % of the molecules in each preparation lie within the limits of DP shown in Table 7, and this forms the basis of the discussion of the heterogeneity of the preparations in an accompanying paper (Whelan & Bailey, 1954). It is manifest that not all the width of the bands can be attributed to deviations from ideal chromatographic behaviour, since the colour play in the zones given by polysaccharides with average DP 34 and 47·5 implies some separation of molecules according to DP within the zone. However, the results obtained with membrane I (last two columns of Table 7) show a bigger spread of DP than the results with the other membranes, and we are inclined to attribute this to a greater departure of the membrane from ideal behaviour, which is in agreement with other anomalies noted above for membranes of this kind. Results with this membrane are accordingly disregarded in the discussion by Whelan & Bailey (1954). These authors also consider our results with the polysaccharide synthesized enzymically from heterogeneous primer.

Applicability of the method

Starch and related polysaccharides. The present results show that the method as so far developed is useful for the characterization of linear dextrins according to their molecular weight; the results with debranched materials suggest that they can simultaneously be separated from substances of higher molecular weight or degree of branching. The range of dextrins of DP 20–250 is satisfactorily handled and the method should therefore be useful in connexion with many problems of enzymic degradation and synthesis in the starch family. It is probably only the evanescence of the colour with iodine which has prevented study of dextrins having DP < 20. Carroll & Van Dyk (1952) have suggested the use of congo red with dextrins in this DP range. Given a suitable detection method and, if necessary, finer-pored membranes, the present method should be useful at and above the DP at which charcoal displacement chromatography and paper partition chromatography cease to be effective.

With the membranes used by us, there were no great differences in the behaviour of amylose and amylopectin, both of which only slowly entered the coarsest membranes. This throws some light on the inconclusiveness of the data of Fouard (1908*a*, *b*, *c*, *d*), who ultrafiltered dispersions of native starch made with boiling water through collodion membranes and eventually concluded that starch is chemically homogeneous but has physical properties which are very sensitive to environmental changes. (See also Taylor & Iddles, 1926.)

Our results seem to establish that potato amylose has a DP considerably greater than 250, since a linear dextrin having this average DP migrated freely away from the origin. They are thus more consistent with the data of Potter & Hassid (1948*a*, *b*, 1951) than with lower estimates.

The 'β-dextrin' from potato amylose did not behave significantly differently from the parent amylose. Dr W. J. Whelan writes: 'Crystalline sweet-potato β-amylase converts only 70 % of potato amylose into maltose (Peat, Pirt & Whelan, 1952), and therefore, as in the case of amylopectin, the amylose molecule would seem to contain anomalous linkages which act as barriers to the endwise progression of β-amylase along the molecule. In amylopectin these anomalous linkages are situated, on average, in the centre of each chain of α-1:4-linked glucose units, and the limit β-dextrin has half the molecular weight of amylopectin. In amylose, however, it would seem that the anomalous linkage is situated at or near the non-reducing chain end, preventing any substantial β-amylolytic

degradation, but in order to account for the degree of conversion into maltose this can apply only to 30% of the molecules; the remainder must be considered free from the anomaly.'

Neither amylopectin nor limit dextrins derived therefrom by the action of β-amylase entered our membranes at any appreciable rate. The products of action on these of R-enzyme gave in all cases a purple-staining fraction unable to enter the membrane rapidly and a freely migrating fraction behaving similarly to the linear dextrins studied. Since in electrophoretic experiments (Mould & Synge, 1954) these preparations also gave material behaving in the same way as linear dextrins, it seems justifiable, pending a study of the behaviour of dextrins having only a few branching points in the molecule, to assume that the migrating materials are in fact linear dextrins. On this assumption their DP distributions have been assessed and throw some light on the structures of the parent polysaccharides and the mode of action of the enzyme. Dr P. N. Hobson writes: 'It was postulated by Hobson, Whelan & Peat (1951) that R-enzyme has a purely hydrolytic function, hydrolysing the α-1:6 branch linkages of amylopectin without the subsequent joining up of the liberated glucose chains by α-1:4 linkages. The results of electrokinetic ultrafiltration analysis are in accord with this hypothesis and also with the finding that R-enzyme does not completely "debranch" amylopectin or limit β-dextrin, as a portion of the products remains unhydrolysable by β-amylase. The structure of amylopectin (and, consequently, the β-dextrin) probably corresponds more closely to the arborescent formula of Meyer (cf. Meyer & Bernfeld, 1940) than to the regular formula of Haworth (cf. Haworth, Hirst & Isherwood, 1937). For discussion see, for example, Peat, Whelan & Thomas (1952). The partial debranching of an arborescent structure would result in some short-chain material (represented by the diffusible fraction of the R-dextrin), linear dextrins of varying chain length, and a molecule containing branch linkages inaccessible to the R-enzyme, which is probably the material which scarcely entered the ultrafiltration membranes. The R-dextrin from a β-dextrin would be expected to yield debranched molecules of lower average chain length than those of the amylopectin R-dextrin, and this is confirmed by the R_F values of the dextrins.'

Finally, since adsorption is presumed to play a part in the separations observed, it should be noted that starch and related polysaccharides have been fractionated on adsorbents by other workers. Cellulose selectively adsorbs amylose from mixtures with amylopectin (e.g. Tanret, 1914; Samec, 1940; Pacsu & Mullen, 1941; Kerr & Severson, 1943; Ashford, Evans & Hibbert, 1946). In view of this, we had hoped that cellulose adsorption chromatograms would fractionate the partial hydrolysis products of amylose. Indeed, paper chromatograms of HCl hydrolysates of amylose developed with 0·2N acetic acid gave long streaks staining pinker with iodine furthest away from and bluer nearest the origin. However, the enzyme-synthesized linear dextrins, now shown to be of rather uniform DP, gave almost equally long streaks on paper under the same conditions. The ineffectiveness of cellulose for chromatography of this series is presumably for the reasons given in the introduction to this paper.

Ulmann (1950*a*, *b*, 1951*a*, *b*) has obtained fractionation of starches on alumina columns. Amylose seems to be selectively adsorbed by ordinary alumina and amylopectin by acid-treated alumina. Similar effects have been described by Fischer & Settele (1953). Ionic interaction of the positively charged adsorbent with the phosphoric ester groups of the amylopectin may play a part. (A similar interaction may enhance the coprecipitation effect with alumina used by Bourne, Donnison, Peat & Whelan, 1949.) The behaviour of the fractions on Ulmann's chromatograms seems to reveal changes in the starting materials not readily detectable by other means. Ling & Nanji (1923) obtained at least partial separation of amylose from amylopectin by adsorption on alumina or colloidal iron. Koval'skiĭ (1947, 1948) has obtained interesting fractionations of glycogens by chromatography on calcium carbonate (see also Koshtoyants & Yanson, 1950; Yanson, 1951).

General. The method has not so far been used preparatively. Difficulties are foreseen in increasing the scale by the use of thicker membranes; satisfactory membranes would be difficult to prepare and temperature differences within them might cause trouble. However, up to 1 mg. polysaccharide has been handled on the present strips (1·3 cm. broad) and some increase of scale could be achieved simply by increasing breadth. It would be well to arrange for fractions to be isolated to run out of the strips electrokinetically, in view of the difficulties noted above in removing polysaccharide from the collodion by mechanical ultrafiltration or diffusion.

Given suitable means of detection, the method should be useful for studying other soluble polysaccharides such as dextrans and fructosans and also intermediate stages in the synthesis and degradation of polysaccharides in general. Substances that do not show a tendency to retrograde from solution are likely to be easier to handle than those here studied. The method should similarly be able to handle other water-soluble neutral polymers, and offers the prospect of obtaining samples of very uniform DP which will be valuable reference substances for physicochemical studies of compounds

of high molecular weight. We are interested also in the possibilities with non-isochemical polymers, especially peptides.

With charged compounds, electrophoretic effects will be superimposed on movement due to the electrokinetic flow. With collodion, only positively charged compounds will enter the membranes. If the compounds are very highly charged, ion-exchange binding in the membrane may be troublesome. For negatively charged substances membranes made from anion-exchange resins or collodion membranes with reversed charge (made in some of the many ways that have been suggested) may prove useful. To extend the method to the large number of polymers that are only soluble in organic solvents which would destroy collodion membranes, it seems promising to try denitrating the collodion (cf. discussion of Mould & Synge, 1952; Fuoss & Mead, 1943; Zhukov, Poddubnyĭ & Lebedev, 1948; Bulgakova & Shafershteĭn, 1951).

SUMMARY

1. In order to circumvent difficulties commonly encountered in chromatographic separation of members of polymeric series, particularly difficulties due to molecular-sieve effects, a procedure has been devised for conducting chromatography inside a strip of collodion ultrafiltration membrane. Flow of liquid in the membrane is produced electrokinetically.

2. The procedure has been evaluated using a series of linear dextrin preparations of known average degree of polymerization (DP) (Whelan & Bailey, 1954). Information has been gained as to the range of DP represented in the various preparations.

3. The separations effected can be explained in terms of normal adsorption of the polysaccharides on the inner surfaces of the membranes. However, ultrafiltration effects prevent molecules greater than a certain size from entering the membrane. Comparisons of adsorption during free diffusion, mechanical ultrafiltration and electrokinetic ultrafiltration suggest that adsorption equilibria at the surfaces may be modified under electrokinetic-flow conditions.

4. Results are reported on velocity of electrokinetic flow in relation to the pore size of the membranes.

5. Several polysaccharide preparations related to or derived from starch have been studied by the new procedure, and estimates of DP range have been made for the cases of linear polymers.

6. The new procedure is discussed in relation to previous work on the chromatography of polysaccharides.

We are grateful to Dr P. N. Hobson, Professor S. Peat, Professor A. Tiselius and Dr W. J. Whelan for helpful advice and criticism and for providing polysaccharide specimens. We also wish to thank Messrs A. Dawson and J. C. Wood for technical assistance.

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The Electrophoretic Mobility and Fractionation of Complexes of Hydrolysis Products of Amylose with Iodine and Potassium Iodide

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(*Received* 15 *April* 1954)

This paper describes some experiments aimed at elucidating the electrophoretic behaviour of polysaccharides related to starch in the presence of iodine and iodide. It has been explained in the previous paper (Mould & Synge, 1954) how these effects came to be observed. The role of iodide in the combination of starch polysaccharides with iodine was studied potentiometrically by Gilbert & Marriott (1948) and by Higginbotham (1949), who give references to earlier work. It has, however, been neglected by most other workers despite the presence of iodide in nearly all of the systems used for studying the starch–iodine reaction. Our experiments also aimed at preparative fractionation of these polysaccharides, and for this purpose the continuous electrophoretic apparatus of Svensson & Brattsten (1949; cf. Grassmann & Hannig, 1950) was used with some modifications in design and operating procedure. Potentiometric and spectrophotometric studies of the behaviour of the isolated fractions with iodine and iodide are described in the following paper (Mould, 1954).

EXPERIMENTAL

Experiments in agar-agar gels

Agar-agar. As explained in the preceding paper (Mould & Synge, 1954) the velocity of electroendosmosis greatly varied with different specimens of agar-agar although the relative velocities of the zones did not differ. No attempt has been made to relate electroendosmosis with composition or origin of the agar-agar; all the later experiments reported here were done with a batch of strip agar-agar, presumably Japanese, supplied before 1939 by British Drug Houses Ltd.

Apparatus and general procedure. This was according to Consden, Gordon & Martin (1946, Method *B*). Gels were set in an enclosed trough 3·8 cm. broad and 0·7 cm. deep, the inlaid zone being 1 cm. broad. The agar was dissolved in boiling water at double the desired final concentration and

the solution, when lukewarm, was mixed with an equal volume of solution of buffer constituents, KI and I_2 also at double the desired final concentration. The mixture was immediately poured into the trough. It was important not to have hot agar and iodine together for longer than avoidable, as I_2 concentration was diminished by chemical reaction. When the gel had set, the inlay zone was poured from solution made in the same way but including the specimen for study (dissolved with the agar). Electrophoresis was not started till about 1 hr. after the inlaying, to ensure thorough setting of the gel. Waiting for longer often gave precipitation of polysaccharide–iodine complexes. A potential gradient of 4–5 v/cm. was used. No special cooling was applied and temperatures of 30–40° prevailed during electrophoresis. Electrodes were, unless otherwise stated, perfused with solution having the same composition as the background gel, with agar omitted.

Appearances in a typical experiment. A run was done using (final concentrations) 2% (w/v) agar-agar, 0·04M sodium acetate buffer (pH 4·7), 0·001M-I_2, 0·00145M-KI and 0·4% (w/v) acid-hydrolysed amylose (Mould & Synge, 1954) (inlay only). During the time between inlaying and commencing the run, some diffusion occurred at the edges of the inlaid zone, the diffused material staining pinker than the purple colour of the body of the zone. Then 5·35 v/cm. was applied, and immediately the cathode edge of the zone became orange-yellow while the anode edge became blue. Resolution was then observed into four separate coloured zones, separated by regions not different in colour from the general background in the trough. After 20 hr. from the start of the run these zones were located as follows, measured from the inlay: yellow-brown, 15·0 cm. towards cathode; orange, 8·5 cm. towards cathode; red, 4·0 cm. towards cathode; blue, 10·0 cm. towards anode. In the early stages of the run, the blue zone became broader than the other zones and did not migrate towards the anode. This is explained by the deficit of free iodine and iodide in the original inlay compared with the background gel due to combination with polysaccharide. As electrophoresis proceeded, I_3^- migrating through the gel towards the anode at its final equilibrium concentration saturated successively the yellow-brown, orange and red zones and last the blue zone. The blue zone was initially further depleted by the faster anionic migration of I_3^-, with the result that it broadened, losing anionic mobility. However, on resaturation by I_3^- moving from the cathode side, a compact zone of characteristic mobility was again formed. Another consequence of the initial undersaturation of the polysaccharide zones was that a colourless zone migrated from the inlay fastest towards the anode, having a velocity which we have assumed to be determined by the mobility of I_3^-, the form in which most of the iodine in the background gel is thought to exist. This colourless zone has been used in determining the mobilities of the different complexes relative to that of I_3^- (see below).

Some of the agar-agar specimens studied, including that used in the experiments now described, gave a faint purple stain when made up in the trough with I_2–KI as described above. This was presumably due to the presence in the agar-agar of some iodine-staining polysaccharide. In the present case, this background stain gave two well-defined boundaries which migrated at different rates away from the anode end of the gel, leaving behind the slower boundary a region of gel devoid of purple stain.

It was noted that, where there were lumps of imperfectly dispersed agar-agar in the gel, blue-staining material was unable to penetrate the lumps and collected on the side of the lump from which it was migrating irrespective of whether this was towards cathode or anode. A clear streak developed on the opposite side of each lump. Red- and orange-staining materials were not affected by the lumps. These effects are attributed to molecular-sieve action.

Effects of varying conditions: relative mobilities of the zones. Essentially similar results were obtained whether amylose hydrolysates made with HCl or salivary amylase (Mould & Synge, 1954) were used. Relative intensities of the different zones varied with the extent of the hydrolysis. *N*-2:4-Dinitrophenylethanolamine (DNPE) could be included in the inlay as a marker of electroendosmotic movement of liquid in the gel (cf. James & Synge, 1951). Its final concentration in the inlay was half-saturation and it did not interfere with the movement of the polysaccharide zones, although the yellow-brown zone partly overlapped the DNPE zone and could not easily be seen. Using 1% (w/v) agar-agar, 0·04M sodium acetate buffer (pH 4·7), 0·0005M-I_2, 0·00145M-KI and salivary amylase-hydrolysed amylose that had been freed from salt by dialysis in cellophan (0·5 mg./ml. in inlay), the zone centres had the following positions after 6 hr. at approx. 4 v/cm.: DNPE, 7·0 cm. towards cathode; orange, 2·0 cm. towards cathode; red, 1·0 cm. towards cathode; blue, 2·7 cm. towards anode; decolorized (I_3^-), 7·7 cm. towards anode. Taking the position of the DNPE zone as giving zero mobility, the anionic mobilities relative to that of I_3^- under the same conditions are thus: blue, 0·66; red, 0·41; orange, 0·34. Doubling the iodine concentration (cf. experiment in previous paragraph) did not markedly affect these relative mobilities. It was not practicable to use still higher I_2 concn., as this led to precipitation of the complexes. It was likewise difficult to do quantitative experiments at lower I_2 concn. on account of the agar–I_2 reaction referred to above, which led to actual I_2 concns. being significantly lower than intended and difficult to control. However, the

general effect of decreasing I_2 concn. while maintaining concn. of KI constant was for the zones to become less intensely coloured and to approach more closely the electroendosmotic flow rate (ascertained using DNPE). The yellow-brown zone was the first to be affected in this way by decreasing I_2 concn. and the blue zone the last. In the absence of I_2 the polysaccharides all migrated at substantially the same rate, corresponding to that of electroendosmotic flow, as revealed by removing the cover from the gel and spraying it with I_2–KI solution at the end of the run. The same result was obtained whether KI was present or absent during the run. The single zone thus observed had in all cases a somewhat orange-staining leading edge and a blue-purple trailing edge, indicative of adsorption or molecular-sieve effects (see Mould & Synge, 1954). However, increasing the agar-agar concn. in the gel to 8% (w/v), while intensifying this effect somewhat, did not lead to practically useful separations, and agar was accordingly abandoned for the electrokinetic ultrafiltration studies.

The effect of I_2 in conferring anionic mobility on the polysaccharides could be conclusively demonstrated by omitting I_2 from the gel, inlay and electrode perfusion fluids in the experiment described above. After running for a suitable time, I_2 was then added to the cathode perfusion fluid. The boundary of a yellow region then migrated into the gel from the cathode end with the velocity characteristic of I_3^- and when this boundary met the polysaccharide zone advancing towards the cathode the characteristic colour play and resolution commenced, the resulting zones migrating as in the original experiment except that the point of origin had been displaced several cm. towards the cathode.

Preparative fractionation by continuous electrophoresis

The colour of the iodine–iodide complexes being a function of the molecular weight or degree of polymerization (DP) of the straight chain polysaccharides (Swanson, 1948; Bailey, Whelan & Peat, 1950), the striking electrophoretic separation in agar gels appeared to offer a possibility of fractionation of the hydrolysis products of amylose into a molecular-weight series. The portion of gel containing the appropriate fraction might be cut out and the polysaccharide allowed to diffuse out into free solution, but the agar tends to disperse into solution and gives components of low molecular weight which are difficult to separate (cf. Gordon & Reichard, 1951). Even if the method was practicable, the amounts that could be handled would be small. For preparative work continuous electrophoretic fractionation has been carried out in an apparatus similar to that devised by Svensson & Brattsten (1949).

Apparatus (*Fig.* 1). The apparatus has been enlarged and modified for large-scale separation of a mixture into fractions differing by 10^{-5} cm.^{2}v^{-1} sec.$^{-1}$ in mobility. The internal dimensions of the Perspex trough were $14 \times 20 \times 0{\cdot}8$ in., the base being slotted with fifty-one grooves, 0·25 in. in depth. (It was subsequently found advantageous to increase the effective depth of the slots by projecting the sides 0·75 in. into the trough so that when the fractionated material entered the slots it was still within the influence of the electric field.) A short length of stainless-steel tubing (int. diam. 1 mm.) is inserted into each groove, the centres being 0·25 in. apart. The outlet tubes are connected by thin-bore Polythene tubing to individual siphons of adjustable height held by clips to a wooden frame, the siphon outlets dripping into collecting flasks. Sheets of plate glass with 1 in. thick Rubazote sponge-rubber gaskets (Expanded Rubber Co. Ltd., Croydon) form cooling troughs through which tap water circulates on either side of the Perspex trough, the whole arrangement being bolted together between two mild-steel frames. Two thin Perspex walls are inserted in the centre of the trough at the upper edge to form a chamber 0·125 in. in width to permit introduction of the mixture to be analysed. Instead of using powdered glass as the stabilizing medium, an improved regularity of flow and diminished adsorption was obtained by filling the trough with grade 12 ballotini beads (English Glass Co. Ltd., Leicester) 0·2 mm. diam. About 16 lb. of glass beads were required to fill the trough. Blockage of the outlet tubes by the medium was prevented by packing the slots in the base with glass wool. For the elimination of gassing and contamination by electrolysis products the electrode chambers were perfused independently of the stabilizing medium. A platinum wire was mounted in the centre of a 0·5 in. diam. Tufnol (Tufnol Ltd., Manchester) tube, 24 in. long, fitted at one end with inlet and outlet arms. The tube had a 0·25 in. milled slot 17 in. long to within 0·5 in. of the base and was placed inside a cellophan dialysis-tubing sac pulled tight over the milled slot and held in position by cotton at intervals along the length. Both electrodes were inserted into the trough before filling it with the glass beads. The performance of the electrodes was satisfactory, although after about 100 hr. electrophoresis at p.d. 350 v and current 200 ma, the cellophan at the cathode tended to become brittle and had to be replaced. They were perfused with dilute alkali and acid at the anode and cathode respectively, the levels being maintained by a Polythene siphoning drainage tube. The level of the buffer solution in the filled trough was kept constant by a constant-head device, the two halves of the trough being connected by two small siphons.

Fractionation procedure. The trough was flooded with 0·04 M sodium acetate buffer (pH 4·8) containing 0·001 M-I_2 in 0·002 M-KI (final concentrations). This concentration of iodine was sufficiently high for complex formation of the short-chain dextrins with iodine. The cathode was perfused with 0·01 M acetic acid containing double the iodine–iodide concentration in the buffer. The presence of iodine was necessary to maintain the iodine concentration in the trough in the region of the cathode, the migration of the tri-iodide ion being high compared with the downward velocity of the buffer flow. The anode was perfused rapidly with 0·01 M sodium acetate in order to remove the high concentration of iodine accumulating at this electrode. The rate of flow through each of the drainage tubes of the trough was arranged to be 0·20–0·25 ml./min., the mixture then taking 2·25 hr. to traverse the trough. A p.d. of 350 v was applied

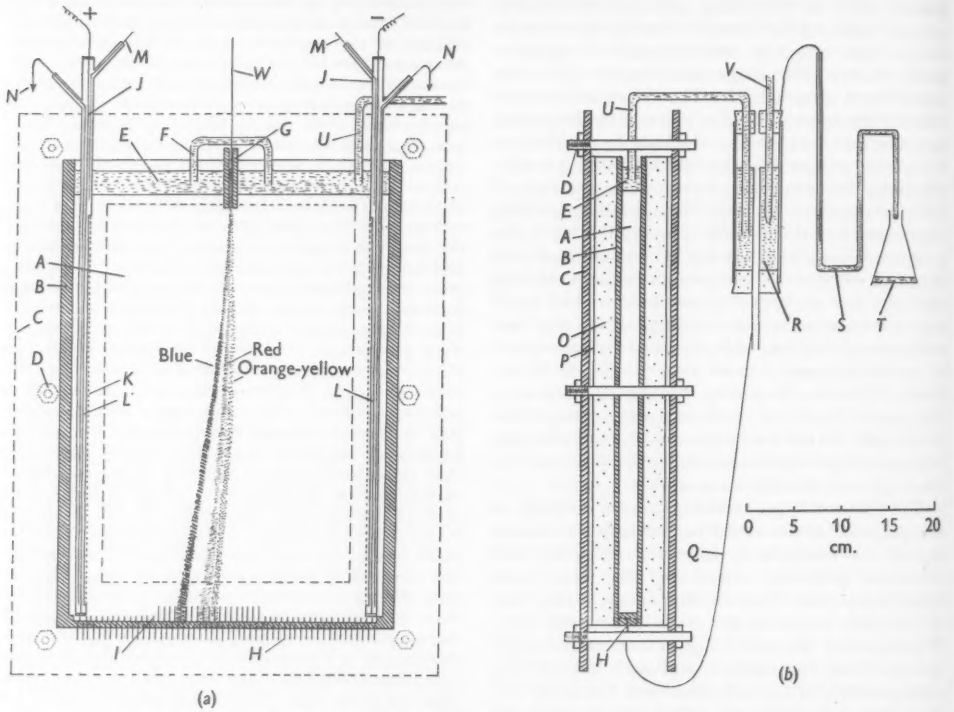

Fig. 1. Preparative electrophoresis cell showing fractionation of hydrolysis products of amylose as complexes with I_2–KI. (*a*) Section through cell seen from front (dotted lines indicate steel frame). (*b*) Section through cell seen from side (electrodes removed). *A*, glass-bead filling; *B*, Perspex trough; *C*, mild-steel frame; *D*, steel bolts; *E*, buffer solution; *F*, siphon connecting two sections of trough; *G*, mixture inlet chamber; *H*, stainless-steel outlet tubes; *I*, grooved base; *J*, electrodes; *K*, cellophan; *L*, platinum-wire electrodes; *M*, electrode perfusion inlet; *N*, electrode perfusion drainage siphon; *O*, Rubazote gaskets for cooling troughs; *P*, plate glass; *Q*, Polythene outlet tubes; *R*, constant-head chamber; *S*, collecting siphon; *T*, collecting flask; *U*, buffer inlet to trough; *V*, buffer inlet to constant head; *W*, mixture inlet siphon.

across the electrodes, producing a current of about 250 mA. The temperature at the centre of the glass-bead packing was 18° under these conditions with tap-water cooling through the external troughs.

The scale of the continuous fractionation was largely determined by the concentration of the mixture. It was found that a solution of polysaccharide more concentrated than 1 mg./ml. retrograded, and precipitation of the iodine complex occurred at the high iodine concentration of the trough buffer, thus setting an upper limit to the concentration of the polysaccharide mixture. Under the flow conditions given above the rate of flow of the mixture was 12 ml./hr. so that, allowing for the time taken to flow through the trough, in a continuous 8 hr. electrophoretic run about 60–70 ml. could be fractionated in a day. The process being continuous, longer runs up to 12 hr. have been made. Solutions of salivary α-amylase-hydrolysed amylose taken to various stages of hydrolysis as determined by 'blue value' (B.V.) (Mould & Synge, 1954) were fractionated under these conditions. The polysaccharide solution was dialysed against distilled water for the removal of strong acid and alkali that would otherwise affect the acidity of the cell buffer in the region of the flowing mixture. Before introduction into the fractionation cell 0·05 M-I_2, 0·1 M-KI was added to the polysaccharide solution to give the same final concentration of iodine as in the buffer.

In a typical fractionation of an enzymic hydrolysate (B.V. 0·56, the mixture with iodine–iodide then exhibiting a blue-purple coloration, a blue complex immediately separated away from a red complex and by the time the base of the trough was reached a distinct background-coloured gap existed between the two bands. The red complex also separated from a slower-migrating yellow-brown complex, although there was not complete resolution. All the bands migrated electrophoretically towards the anode, the blue complex having the highest apparent mobility. The flow lines of the migrating complexes tended to be slightly curved due to the unbalanced small electro-

endosmotic flow (Svensson & Brattsten, 1949) in the acetate buffer. The width of the bands increased by diffusion during passage through the cell. The blue complex emerged from two outlet tubes separated by a gap of one or two tubes from the red complex also spread out over two outlet tubes. The fractions were collected continuously. The output from intermediate tubes where mixing might have occurred due to slight variations in the width or displacement of the bands during the continuous flow was rejected. This was especially necessary for the satisfactory fractionation of the slower-moving and incompletely resolved red and brown complexes.

In acid-hydrolysed amylose the blue-staining band appeared to be much broader than that obtained from α-amylase hydrolysates of similar B.V. There was also a larger proportion of red and orange-yellow components. In this broad blue-staining band there was evidence of an appreciable spread in mobility across the band as a repeat electrophoretic run of the intermediate mixed blue and red fraction did not give such a wide spread in the blue band, the faster-moving blue components being presumably absent.

Isolation of dextrins. The dextrins, as collected from the outlet tubes of the cell, were present as complexes with I_2–KI in the presence of sodium acetate buffer and excess I_2–KI. The iodine was destroyed by titration with slight excess of sodium thiosulphate, using the disappearance of colour of the complex as indicator. The clear solution was then dialysed in cellophan against distilled water for 2 days and finally concentrated *in vacuo* to *ca.* 50 ml. Last traces of salts were removed by electrodialysis in a four-compartment cell (Theorell & Åkeson, 1942; Synge, 1951). The pH of the specimen compartment usually remained between 4 and 5 or was so maintained by addition of dilute NH_3 or acetic acid until a constant current of about 5 mA with a p.d. of 230 V was reached. Some of the fractions tended to become cloudy and precipitated at these very low salt concentrations at the completion of electrodialysis. The salt-free solution was then concentrated *in vacuo* to 5 ml. and the dextrin precipitated by addition of 4 vol. ethanol. The precipitate was repeatedly washed with ethanol on the centrifuge, and finally dried *in vacuo* at room temperature yielding a white amorphous powder. The products were stored over P_2O_5.

A quantitative estimation of the yield of the various electrophoretic fractions in relation to the initial amount of amylose before hydrolysis was not possible as loss of material unavoidably occurred. Mixing of the intermediate collected fractions and their rejection has been mentioned above; abnormalities during an electrophoretic run have on occasion prevented fractionation of part of the original hydrolysate; there may also be incomplete precipitation of the salt-free solutions by ethanol during the extraction procedure and losses in dialysis. The purity of the fractions, i.e. the absence of coloured complexes other than the one collected, was assessed by electrophoretic separation using the agar-gel technique described above. Satisfactory fractions gave a single zone. The difference in movement towards anode and cathode of the blue- and red-staining complexes respectively afforded a sensitive test of contamination. In one run with 42·5 mg. amylose (α-amylase hydrolysis to B.V. 0·28; made to 50 ml. for fractionation) blue and red bands of about equal intensity were seen. On working up the collected fractions 12·2 mg. of 'blue' material, 15·4 mg. of 'red' material, and 2·2 mg. of 'mixed' fractions were isolated. The orange-yellow staining fractions were not estimated nor were the very short-chain fragments of hydrolysis. The latter are too short to form stable complexes with iodine and are thus uncharged.

Estimation of chain lengths in electrophoretic fractions. The spread of DP in the fractions was estimated by electrokinetic ultrafiltration analysis in collodion membranes (see Mould & Synge, 1954). After iodine staining of the collodion strip the red material gave a uniform pink coloration, while the front of the zone of the blue material had a slight purplish tinge changing to a uniform blue throughout the rest of the zone. A mixture of the two fractions gave a single zone with no gap in the spread of colour. The purple colour exhibited by the intermediate range, DP 40–50 (cf. Whelan & Bailey, 1954) is not observed during electrophoretic separation in agar gels or in the stabilized glass-bead medium, all the material migrating as a single blue zone. The orange-yellow staining fraction could not be studied by the electrokinetic ultrafiltration procedure owing to the low sensitivity of the iodine staining. A chromatographic separation of the achroic fraction on paper using an amyl alcohol–pyridine–water mixture as solvent (Jeanes, Wise & Dimler, 1951) showed the presence of short-chain dextrins (DP <10).

DISCUSSION

Electrophoretic fractionation of polysaccharides

Many naturally occurring polysaccharides do not contain ionizing groups and thus, being uncharged in solution, cannot be fractionated by electrophoretic procedures. In fact this electrical neutrality provides a satisfactory basis for the separation of mixtures of charged protein material and carbohydrates (reviewed by Svensson, 1948), and for the isolation of the protein moiety from protein–polysaccharide complexes. There are few examples of the fractionation of uncomplexed polysaccharides. Samec and others (reviewed by Samec & Blinc, 1938; see also Dahl, 1940; Hopkins, Stopher & Dolby, 1940) have fractionated native tuber starches into amylopectin and amylose components by electrodecantation in a vertical field, the phosphate groups esterified to the amylopectin (Posternak, 1935, 1951; Schoch, 1942) causing migration to the anode while the uncharged amylose remains in the supernatant solution. The fractionation is complicated by the presence of charged impurities and the amylose tends to retrograde and precipitate. Phosphate groups are only loosely bound to cereal starches (Posternak, 1935, 1951; Schoch, 1942) and the amylose and amylopectin components are not electrophoretically separable. Saric & Schofield (1946) studied the dissociation of carboxyl and hydroxyl groups of cellulose, starch and other polysaccharides in strongly alkaline solutions and found that in 5N alkali at pH 14·7 it may be regarded as analogous to the dissociation of a weak polybasic acid. The high current density, ohmic heating and increase in diffusion introduce difficulties in the application of this interesting behaviour for the electrophoresis of polysaccharides.

Isherwood (1949), however, reported that various cellulose-type cell-wall polysaccharides migrate with different mobilities under these alkaline conditions and that the formation of one boundary was a strong indication that only one polysaccharide was present. These experiments have been repeated by Colvin, Cook & Adams (1952) using polysaccharide mixtures in M alkali with a failure to obtain repeatable electrophoretic patterns. They suggest that the adsorption of the hydroxyl ions on the large neutral molecules would mask any differences in the ionization constants of the hydroxyl groups, and they demonstrated this effect in the separation of sodium alginate from laminarin.

Electrophoretic techniques have been successfully applied to polysaccharides containing ionizing groups such as the polyuronic acids (Säverborn, 1945) and the mucopolysaccharides heparin, chondroitin sulphate, mucoitin sulphate, hyaluronic acid (Wolfrom & Rice, 1947; Gardell, Gordon & Åqvist, 1950). A spread of mobilities with no definite separation, presumably indicating heterogeneity, has been observed with carrageenin (Cook, Rose & Colvin, 1952). The enzymic and acid demethylation of pectin has been compared in terms of the spread of electrophoretic mobilities (Speiser, Copley & Nutting, 1947). The separation of amylose, amylopectin, dextran, glycogen, heparin, hyaluronic acid, levan, yeast mannan, synthetic polyglucose, and starch by paper electrophoresis in barbiturate buffer has been claimed by Greenway, Kent & Whitehouse (1953). For materials other than heparin and hyaluronic acid this is unlikely to be a true electrophoretic separation and is more probably due to a combination of adsorption and electro-endosmotic flow which these authors ignored. Amylopectin moved to the cathode with the electro-endosmotic streaming as a discrete spot while the amylose was strongly adsorbed by the filter paper and remained at its initial position. The preferential adsorption of amylose by cellulose is a well-established method for the purification of amylopectin. The zone sequence is thus inverted from that in the truly electrophoretic separations of amylose and amylopectin referred to above.

Norberg & French (1950) analysed electrophoretically a mixture of Schardinger α-, β-, and γ-dextrins dissolved in 0·087 M potassium iodide. No explanation of the difference in mobilities was given (cf. Cramer, 1951). Norberg & French (1950) also described the oxidation of short-chain reducing oligosaccharides to the corresponding acids by alkaline hypoiodite, which after conversion into the potassium salts could then be separated electrophoretically.

It would appear that a more extensive application of electrophoretic fractionation techniques to polysaccharides is only possible if attention is directed towards the formation of charged complexes. The separation of sugar–borate complexes by paper electrophoresis has been developed (Consden & Stanier, 1952; Jaenicke, 1952; Michl, 1952; Micheel & van de Kamp, 1952) and has been extended for the study of related compounds (Foster, 1952*a*, *b*; Foster & Stacey, 1953). The mechanism of borate-complex formation in relation to methylated sugars, cyclitols, glucosaminides, glycosides and disaccharides has been discussed (Foster, 1953; see also Woodin, 1952). Fractionation of the electronegative cellulose–cupriethylenediamine complex into two migrating boundaries characteristic of the dissolved cellulose has also been reported (Adams, Karon & Reeves, 1951).

The decrease in electrophoretic mobility of the iodine–iodide complexes of the hydrolysis products of amylose with decreasing chain length has led to possibilities of fractionation according to molecular weight.

Electrophoretic fractionation of hydrolysis products of amylose as iodine–iodide complexes

The electrophoretic fractionation separates the hydrolysate into (1) a scarcely staining zone DP < 10, (2) an orange-staining zone DP 10–25, (3) a red-staining zone DP 25–40, and (4) a blue-staining zone DP 40–130, the upper limit depending on the degree of hydrolysis. The zones are separated by gaps in which polysaccharide is absent.

A decrease in the longest chain length in the hydrolysate with increasing hydrolysis is observed (Table 1). A series of separations of α-amylase

Table 1. *Estimated chain length in electrophoretic fractions from amylose hydrolysed with α-amylase*

DP was estimated by electrokinetic ultrafiltration (Mould & Synge, 1954).

Degree of hydrolysis (B.V.)	Fraction	DP
0·56	Red	25–40
	Blue	40–120
0·42	Red	27–42
	Blue	40–90

Table 2. *Change in proportion of blue and red material during α-amylase hydrolysis of amylose*

Yields reported are from preparative electrophoretic runs on various hydrolysates.

Degree of hydrolysis (B.V.)	Blue material (mg.)	Red material (mg.)	Blue/red
0·1	2·4	4·0	0·6
0·25	44	66	0·66
0·28	12·2	15·4	0·79
0·34	50	37	1·35
0·39	79	34	2·3
0·45	81	44	1·84
0·57	55	16	3·3

hydrolysates of decreasing B.V. has been made and the relative amounts of blue- and red-staining material estimated (Table 2). As would be expected, the ratio of blue material to red material falls with increasing degree of hydrolysis, although it is interesting to observe that even at a low B.V. (0·2), where the iodine-staining colour of the hydrolysate is predominantly red, there is still a comparatively high amount of the blue material still present. The greater spread of chain lengths observed in the acid hydrolysate would be expected as a result of a more random breakage of the polyglucosidic chains during acid hydrolysis compared with α-amylase hydrolysis (cf. Meyer & Gonon, 1951).

Structure of the iodine–iodide complex and electrophoretic mobility

In a consideration of the formation of a charged complex of the dextrin with iodine–iodide solution the conception of a helical structure of the polysaccharide molecule with an inner core of resonating iodine atoms (Hanes, 1937; Freudenberg, Schaaf, Dumpert & Ploetz, 1939; Bear, 1942; Rundle & Baldwin, 1943; Baldwin, Bear & Rundle, 1944; Stein & Rundle, 1948) is extended to include iodide ions as supplying the resonating electrons (Gilbert & Marriott, 1948).

A limited study (Mould, 1954) of the potentiometric titration of the electrophoretically separated fractions with iodine has been made using the method of Gilbert & Marriott (1948) (cf. Bates, French & Rundle, 1943; Dvonch, Yearian & Whistler, 1950). The characteristic blue coloration increases in intensity during the formation of the complex $3I_2$–yI^-. In the case of the red-staining material the complex $2I_2$–$y'I^-$ is formed. The evaluation of y and y' has not been attempted owing to the lengthy experimental work involved.

The ultraviolet and visible absorption spectra of the fractions when in complex formation with iodine have also been examined (Mould, 1954), and it has been shown that in the longer helices giving a blue coloration there is probably a strong polarizing effect of the I^- ions on the I_2 molecules in the chain, with a gradual extension in the average separation between the iodine atoms in the core as the length of the helix increases. With decreasing chain length and shift of the absorption peak in the visible range to shorter wavelengths, there is a tendency towards the formation of an I_3^--type complex.

Without further extensive potentiometric studies of the complex structure, which may be complicated by surface adsorption of iodine (Mould, 1954), one can only speculate as to the probable nature of the complex. The supposition is made here that the colour and mobility of the blue migrating complexes are determined by the presence of a $(3I_2$–$2I^-)_n$ grouping (similar to the structure of an amylose–iodine–iodide complex reported by Gilbert & Marriott (1948)) and that the colour and mobility of the red migrating complex are determined by an $(I_3^-)_n$ grouping. A length along the helix of 8 Å per single turn of 6 glucose units is taken from the data of Dombrow & Beckmann (1947). The ratio of the length of the iodine–iodide core to the length of the helix increases with DP so that the net charge and amount of iodine absorbed will increase rapidly with DP. A linear array of three $(3I_2$–$2I^-)$ groups may exist in a helix of DP 72, length 96 Å, giving a charge/mass ratio $4{\cdot}1 \times 10^{-4}$. The average separation between the iodine atoms in such a linear resonating array is 3·1 Å (Hach & Rundle, 1951; Cramer, 1951; Cramer & Herbst, 1952), although such stable conditions are probably not attained in the present case. The length of the $9I_2$–$6I^-$ complex considered would therefore be < 70 Å. Allowing for a decrease in diffusion coefficient with increasing DP in relation to the increasing net charge, it is conceivable that the electrophoretic mobilities may only extend over a narrow range. In the red-staining dextrins a loosely bonded linear array of two I_3^- ions may exist in a helix of DP 36, length 48 Å, giving a charge/mass ratio $3{\cdot}0 \times 10^{-4}$. Assuming a length 5·92 Å for the I_3^- ion (Mooney, 1935) and 3·0 Å for the separation between the two ions, the distance between the extreme atoms of the $2I_2$–$2I^-$ complex is 14·8 Å. The mobility of a short dextrin complex is reduced therefore by virtue of the very short iodine–iodide core that can be established.

Whether or not this speculation proves correct the apparently abrupt change in the mobility and colour of the iodine–iodide complex over a narrow range of chain lengths at approx. DP 40 is striking. Failure to isolate material of intermediate chain length giving a purple stain in free solution raises the question of whether the purple stain (observed with partial hydrolysis of amylose and with amylopectin, etc.) corresponds to a true complex or a mixture of red and blue complexes arising from the competition of the polysaccharides for iodine and iodide ions. The purple coloration of partially hydrolysed amylose could obviously arise from a mixture of red and blue complexes and it has also been shown (Whelan & Bailey, 1954) that in an enzyme-synthesized polyglucose of average DP 45 there would be a statistical spreading of the chain lengths sufficient for the formation of both red and blue complexes. A definite conclusion as to the colour of the complex over a narrow range of DP could be made by refinement of the electrokinetic ultrafiltration procedure (Mould & Synge, 1954) to give material of narrow DP range. It is suggested that in this critical range around DP 40, there may

be a sudden change in the type of complex dependent on the formation of a longer iodine–iodide core, so that, according to the availability of iodine, the dextrin may form either a red or blue complex.

It was not possible to derive values for the mobility of the complexes from their movements in the preparative cell. Mobilities could be calculated from the agar-gel experiments but were only strictly applicable for electrophoretic movement through the gel medium itself. There was also no control of temperature during the separation. For these reasons only the relative mobilities of the various complexes have been considered.

The potentiometric titration of the fractionated dextrins with iodine–iodide solution (Mould, 1954) has shown that the minimum concentrations of iodine required for saturation in 10^{-3}M-KI are $1{\cdot}0\times10^{-5}$M, $3{\cdot}2\times10^{-5}$M, and $15{\cdot}0\times10^{-5}$M respectively for the blue, red and orange complexes. During fractionation in the preparative cell the complexes were therefore under saturation conditions, and increasing the iodine concentration above 0·0005M in the agar-gel experiments did not in fact affect the relative mobility of the complexes. However, as observed in these experiments, progressively decreasing the iodine concentration will obviously prevent the formation of the yellow-brown complex first and the blue complex last. Gilbert & Marriott (1948), in their potentiometric studies of amylose–iodine complexes, found that the blue colour of the amylose complex developed when the ion ($3I_2$–$2I^-$) was present but at lower iodine concentrations a small quantity of another ion suggested as ($2I_2$–I^-) is formed. The presence of ions of lesser charge in low iodine concentration could also be inferred from the potentiometric titration curves of the fractionated dextrins considered here (Mould, 1954). The broadening of the mobilities and change in direction of migration of the blue complex during the initial stages of electrophoresis in agar gels provides further evidence for the existence of these unsaturated complexes.

SUMMARY

1. Partial hydrolysates of amylose are resolved into four separate coloured zones by zone electrophoresis in agar jelly in the presence of iodine–iodide. The effects on the resolution of varying the iodine–iodide concentration are described. *N*-2:4-Dinitrophenylethanolamine was used as a marker of electroendosmotic movement.

2. Continuous electrophoretic fractionation of α-amylase-hydrolysed amylose in the presence of iodine–iodide has been carried out in an apparatus similar to that of Svensson & Brattsten (1949).

3. Four fractions have been isolated in a salt-free state, viz. material from (*a*) a non-staining zone having degree of polymerization (DP) <10; (*b*) an orange-staining zone DP 19–25; (*c*) a red-staining zone DP 25–40; (*d*) a blue-staining zone DP 40–130 (the upper limit depending on the degree of hydrolysis).

4. With improvement of technique fraction (*d*) could probably be further fractionated. The method is applicable to the study of enzymic breakdown of high-molecular starch-like polysaccharides and possibly to the fractionation of polydisperse substances such as amylopectin or glycogen.

5. Possible structures of the various iodine–iodide–dextrin complexes are discussed, and it is suggested that there is a sudden change in the type of complex formed at DP *ca.* 40. The purple-staining complexes formed by dextrins in the range DP 40–50 may be a mixture of red- and blue-staining complexes.

We are grateful to Dr G. A. Gilbert, Professor A. Tiselius and Dr W. J. Whelan for critical discussions. We wish to thank Messrs A. Dawson and J. Wood for technical assistance.

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Potentiometric and Spectrophotometric Studies of Complexes of Hydrolysis Products of Amylose with Iodine and Potassium Iodide

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(*Received* 15 *April* 1954)

The formation of coloured complexes in starch–iodine–iodide solutions is of interest in relation to the structure of starch components and also as to the nature of the molecular linkage and optical absorption in iodine–iodide chains. The establishment of the helical structure of the amylose fraction of starch (Hanes, 1937; Freudenberg, Schaaf, Dumpert & Ploetz, 1939; Bear, 1942; Rundle & Baldwin, 1943; Spark, 1952) and the potentiometric titration of amylose with iodine (Bates, French & Rundle, 1943; Gilbert & Marriott, 1948; Higginbotham, 1949) have led to the idea of a collinear core of iodine and iodide molecules arranged end-to-end inside the amylose helix. Recent advances in the enzymic synthesis of a homologous series of straight-chain polysaccharides consisting of glucose residues in α-1:4 linkage (Cori & Cori, 1939; Whelan & Bailey, 1954) have opened up a possible extension of the potentiometric and other methods of investigation for the study of the changes in the iodine–iodide structure and its relation to the change of colour and optical absorption spectra of the complex (Bailey, Whelan & Peat, 1950).

The potentiometric titration of amylose (Bates *et al.* 1943) and of fractionated preparations of hydrolysed amylose (Dvonch, Yearian & Whistler, 1950) with iodine have shown that the bulk of iodine in the complex is absorbed at constant activity, followed by a slow increase, attributed to surface absorption, with the continual increase in the concentration of free iodine as the titration proceeds. For a straight-chain amylose less than *ca.* 100 glucose units it is not possible to distinguish clearly between surface absorption and complex formation. (The order of chain length stated by Dvonch *et al.* is based on earlier possibly inaccurate spectrophotometric estimates of chain length by Swanson, 1948: cf. Bailey *et al.*, 1950; Bailey, 1952.) The iodine activity required for complex formation is a function of the molecular weight of the amylose, being lower for samples of higher molecular weight. Long helices tend to be filled before short ones can absorb appreciably, the stability of the iodine–

iodide core increasing with length. An explanation of this effect has been discussed by Stein & Rundle (1948) in terms of the polarization of a chain of iodine molecules by the electrostatic field induced along the axis of the helix. Baldwin, Bear & Rundle (1944) have reported a fall in the maximum iodine content of the helices with increasing iodide concentration due to a replacement of iodine molecules with iodide or triiodide ions. They suggest that, the adsorption of the iodine molecules attached to the ends of the helix being weak, the activity as determined by potentiometric measurements increases before the last possible iodine has entered the helix, causing the apparent amount of iodine adsorbed to be less than that determined by spectrophotometric titration. In this latter method all the iodine is assumed to be in complex formation if it contributes to a rise in the optical absorption of the solution over that of an iodine–water solution. In calculating from the spectrophotometric data the amount of iodine adsorbed, the contribution of the free iodine in solution has been neglected. This error is slight in the case of amylose but for shorter straight-chain polysaccharides the increase in free-iodine activity necessary for complex formation increases the contribution appreciably.

Gilbert & Marriott (1948) have studied the adsorption isotherm of amylose in the region of very small percentage adsorption over a range of low iodide concentration (10^{-3}M–10^{-4}M) using a differential potentiometric method. Under these conditions the amylose complex is comprised mostly of groups of the type ($3I_2$–$2I^-$), the number of iodine molecules in the complex increasing with increase in iodine activity. If the iodide concentration is increased the ratio of iodine molecules to iodide ions incorporated tends towards 1, i.e. $(I_3^-)_n$, accompanied by the appearance of a slightly purple tinge to the blue of the complex. Gilbert & Marriott have suggested that forms of starch or degraded starch which form red or purple complexes with iodine are unable to stabilize a long chain of iodine and iodide atoms and that an extension of the method of iodine titration may reveal in such cases complexes of less than eight atoms of iodine or iodide ions per molecule. It is interesting to compare these observations with those of Godina & Faerman (1950) on the crystalline complexes formed only in the presence of iodide ions by an ethanolic solution of iodine with quinine bisulphate. The colour of these compounds varies from red through purple to blue as the I_2:HI ratio changes from 1 to 2. A similar shift of the absorption peak to longer wavelengths with increasing iodine concentration is also observed with polyvinyl alcohol films. As Rundle, Foster & Baldwin (1944) have stated, the general appearance of pleochroism suggests the existence of a parallel array of iodine molecules in all these complexes, amylose solutions being unique by virtue of a single long molecule forming a helical envelope for the iodine molecules, while the other crystalline substances consist of an aggregate of orientated crystals with iodine adsorbed between the molecules (cf. Meyer & Bernfeld, 1941).

Electrophoretic fractionation of α-amylase-hydrolysed amylose in the presence of I_2–KI solution has been carried out on a continuous preparative scale. The degree of polymerization (DP) of the blue-, red-, and orange-staining fractions ranged from 90 to 40, 40 to 25, 25 to 10 respectively (Mould & Synge, 1954). An attempt towards the characterization of their iodine–iodide complexes has been made by means of potentiometric titration with iodine and observations on the absorption spectra. These measurements are of an exploratory character only, but a critical review of the encouraging results and of certain technical difficulties that have arisen seems appropriate in view of the possibility of similar investigations being extended to enzyme-synthesized straight-chain polysaccharides. The red-staining fraction is no more, and probably less, heterogeneous than preparations so far obtained by direct synthesis of a mixture of similar average DP (Whelan & Bailey, 1954).

METHODS

Potentiometric titrations with iodine

Titrations of the blue- and red-staining fractionated polysaccharides were carried out in the same differential potentiometric-titration apparatus, the technique being essentially similar to that described by Gilbert & Marriott (1948). Two solutions of KI, buffered to about pH 6, one containing the polysaccharide, were joined by a salt bridge and iodine solution was added to each in such quantities that the potential between two platinum electrodes immersed in the respective solutions remained less than 0·05 mv.

Fractionated blue-staining material (20·0 mg.) was dispersed in 4 ml. N-NaOH at room temperature, neutralized with 5·2 ml. N-H_3PO_4 and made up to 100 ml. of solution. Of this solution 90 ml. were made up to 1 l. with KI solution to give final concn. 0·001 M-KI in one of the cells (final pH 6). An identical solution (without polysaccharide) was prepared for the titration cell. Both cells were brought to 20°, the electrodes and salt bridge inserted, and 0·00532 M-I_2, 0·01 M-KI added in small increments to both cells from an Agla micrometer syringe (Burroughs Wellcome and Co.). Following the addition of iodine to the cell containing polysaccharide, the titration cell was balanced to within 0·05 mv, the conditions of balance becoming constant after 10 min., or longer, as the iodine activity increased. A similar procedure was followed for the red-staining material, except that 50·0 mg. polysaccharide were used in the preparation of the solutions.

Owing to the high concentration of iodine required before appreciable iodine adsorption takes place it was necessary to add comparatively large amounts of I_2–KI solution to the cells, with a consequent appreciable increase in KI concen-

tration and volume. Corrections have been applied for these cumulative effects on the balance condition in calculating the free-iodine concentration in the polysaccharide cell.

The sensitivity of detection of the null point is very much lowered at high iodine concentration. For accurate work it would therefore be necessary to use an electrometer circuit capable of detecting a potential difference of the order 0·01 mv (cf. Anderson & Greenwood, 1953). In order to minimize the effect of increasing KI concentration with addition of iodine the titration would have to be performed in stages, i.e. starting at an arbitrary point in complex formation with the initial iodide concentration adjusted to 0·001 M. A titration at a lower iodide concentration, e.g. 0·0001 M, would require a complete preliminary titration and then even more careful repetition over very short ranges of iodine activity at the fixed iodide concentration.

The ultraviolet absorption spectrum of amylose–iodine–iodide complex

Amylose (alumina–thymol precipitated, 10 mg.) from potato starch was dispersed on gentle warming in 1 ml. N-NaOH, the solution neutralized by N acetic acid and made up to 50 ml. at pH 6·0. Samples (5 ml. 1 mg. amylose) were diluted to 25 ml., a requisite amount of 0·0032 M-KI being added to make the final solution 0·001 M in KI after the addition of 0·30, 0·25, 0·20 or 0·15 ml. of a solution of 0·2% I_2 in 1% KI to the samples covering a range of iodine concentration from $9{\cdot}46 \times 10^{-5}$ M to $4{\cdot}73 \times 10^{-5}$ M. The flasks were shaken for 7 min., allowing equilibrium conditions of adsorption to be reached, and an absorption curve plotted from 260 to 400 mμ. in a 1 cm. cuvette on a Beckman DU Quartz Spectrometer. The same time was allowed for each plot so that at any particular wavelength each solution had been under the same conditions since the addition of iodine.

The absorption curves of identical solutions under comparable conditions were plotted from 400 to 700 mμ. Absorption curves from 260 to 400 mμ. for iodine solutions covering a range of concentration of iodine from $9{\cdot}46 \times 10^{-5}$ M to $1{\cdot}81 \times 10^{-5}$ M in 10^{-3} N-KI were also plotted.

The whole experimental procedure was repeated using solutions containing 2 mg. amylose in 25 ml. with iodine concentrations ranging from $12{\cdot}6 \times 10^{-5}$ M to $4{\cdot}73 \times 10^{-5}$ M.

RESULTS

Potentiometric titrations

Representative potentiometric titration curves of adsorbed iodine/mg. polysaccharide against the concentration of free total iodine are shown in Fig. 1, with the change in iodide concentration as titration proceeds indicated. An amylose curve (Gilbert & Marriott, 1948) is included for comparison. The increase in iodine activity with decrease in chain length required for maximum adsorption of iodine is immediately obvious. The gradual increase in iodine adsorption shown by both the blue- and red-staining polysaccharides is probably due to surface adsorption of iodine molecules (Dvonch *et al.* 1950). The tendency for both

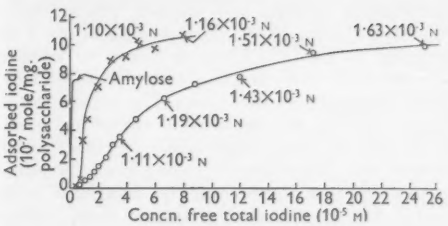

Fig. 1. Titration of fractionated dextrins with iodine in 0·001 M-KI: ×, blue-staining fraction (DP 40–90); O, red-staining fraction (DP 25–40). The change in iodide concentration is indicated. An amylose titration curve is also shown.

curves to reach about the same maximum height may suggest that maximum surface adsorption may then have been attained. The plot of log (adsorbed iodine) against log (total iodine) is drawn in Fig. 2 for the lower portion of the titration curves where there is less interference from this masking adsorption. The KI concentration can be regarded as constant in this region, and following Gilbert & Marriott's treatment the number of molecules (x) of iodine in the complex is given by the relation

$$x = \frac{\mathrm{d} \log a}{\mathrm{d} \log [I_2]},$$

where a = number of moles of iodine bound in complex/g. polysaccharide. In the case of the blue-staining polysaccharide, it is seen that an initial slope corresponding to $x = 2$ changes at higher concentration to $x = 3$. The characteristic blue-complex coloration is first faintly visible when the iodine activity corresponds to the change in slope, thereafter gradually increasing in intensity. Over this range of activity complex formation involving three molecules of iodine would appear probable. Later, as shown by the non-linearity of the log/log plot, this figure falls off with increasing iodine activity and is presumably affected by surface adsorption. The change $x = 2$ to $x = 3$ is similar to that observed in the early stages of blue-complex formation with amylose (Gilbert & Marriott, 1948). The apparent absence of higher complexes might be expected as the shorter helices are unable to stabilize such a long iodine–iodide chain as amylose, but, considering the heterogenity of the fraction and other factors, the experimental evidence is insufficient to provide a conclusive argument.

For the red-staining polysaccharide, the initial slope corresponds to $x = 1$, changing at higher concentrations to $x = 1{\cdot}8$. Again the appearance and intensification of the colour occurs along the second linear portion of the log/log plot. Titration of another sample, prepared from solution by butanol

precipitation instead of ethanol precipitation (Mould & Synge, 1954), gave a slope of $x = 1{\cdot}15$ changing to $x = 2{\cdot}22$. There is reason to believe (Dvonch *et al.* 1950) that the butanol complex aids helical formation of the chains. The experimental values therefore suggest that the complex formed by the red-staining straight-chain polysaccharides contain two molecules of iodine/molecule polysaccharide. This figure may be compared with $x = 1{\cdot}2$ for amylopectin saturated with iodine (Hybart, 1952) as indicative of a difference in the mechanism of complex formation encountered in branched-chain polysaccharides.

The evaluation of the amount of iodide ion in the complex by varying the iodide concentration has not been attempted. This so far can only be inferred qualitatively from the electrophoretic behaviour of the fractions (Mould & Synge, 1954), since the negative charge and mobility in an electric field of the polysaccharide in iodine–iodide solution presumably originates from the iodide ion content, except in so far as the net charge may be modified by interaction in the collinear chain of I_2 molecules and I^- ions.

Owing to experimental difficulties in controlling the iodide concentration no detailed investigation of the orange-staining complex was possible. The minimum concentrations of iodine required for saturation in 0·001 M-KI are $1{\cdot}0 \times 10^{-5}$ M, $3{\cdot}2 \times 10^{-5}$ M and about $15{\cdot}0 \times 10^{-5}$ M respectively for the blue, red and orange complexes.

The absorption spectra of amylose–iodine–iodide complexes

The change in colour from blue to red or the shift of the absorption band to shorter wavelengths during the hydrolysis of amylose has been shown to bear a relationship to the average chain length of the straight-chain polyglucosides present (Swanson, 1948; Bailey *et al.* 1950), although it would appear that a measure of the wavelength of maximum absorption can only be used as a rough guide to the average molecular weight. The extinction coefficients at the absorption peaks also increase with increasing chain length and higher stability of the iodine complex. With branched-chain polysaccharides of the amylopectin type, if the concentration of iodine is sufficiently high, iodine can be adsorbed to the same extent as on amylose mainly by surface adsorption of single molecules or triiodide ions. Higginbotham (1949) has divided the absorption spectrum of amylopectin and glycogen complexes into two components, one with a peak at 540 mμ. due to a short-chain helical complex and the other with a peak of the order of 400 mμ. assumed to be due to adsorbed triiodide ion.

From a review of the literature it would appear that little attention has been given to the near-visible (200–400 mμ.) ultraviolet absorption spectra of the polysaccharide–iodine complexes. The accuracy of measurement in this region of the spectrum is hampered by the large correction which

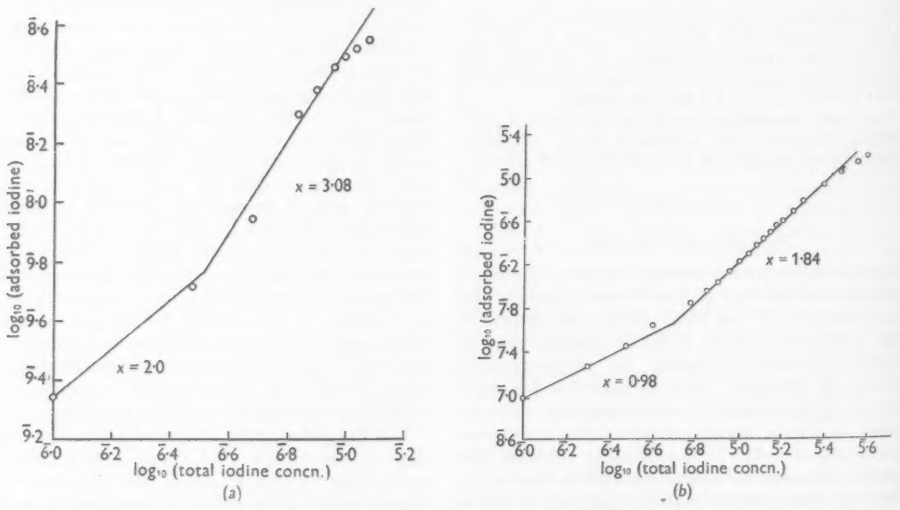

Fig. 2. Plot of $\log_{10}$ (adsorbed iodine) against $\log_{10}$ (concentration of total iodine). Units and conditions as in Fig. 1. (*a*) Blue-staining fraction; (*b*) red-staining fraction.

must be made for the iodine blank and the wide range of concentrations of iodine, iodide, and polysaccharide used by various workers has led to confusion. Iodine in aqueous iodide solution exhibits three well-defined absorption bands at 226, 287 and 353 mμ., the last two being attributed to the triiodide ion (Awtrey & Connick, 1951). The heights of these bands are considerably modified by the presence of polysaccharide (Lampitt, Fuller & Goldenberg, 1941) and Bebbington, Bourne & Wilkinson (1952) have reported the complete suppression of the 287 mμ. band with a slight enhancing of the 353 mμ. band by amylose (see Cramer, 1951). If the amount of free iodine and iodide in the solution is known there is little difficulty in estimating the contribution of the unadsorbed iodine to the absorption spectrum and this can then be subtracted from the observed total spectra to determine the contribution of the absorption spectra of the iodine–polysaccharide complex. A comparison with the absorption spectra of free iodine in solution is likely to be of interest in view of the various possibilities as to the nature of bonding of iodine in the complex.

If the iodine concentration is sufficient to saturate the amylose, the absorption peak in the visible (640–660 mμ.) should remain of constant height. A slight falling off was observed at the lowest concentration of $4{\cdot}73 \times 10^{-5}$ M-I_2. Allowing for a concentration of $0{\cdot}2 \times 10^{-5}$ M-I_2 (Gilbert & Marriott, 1948) to be necessary for saturation, it may roughly be inferred that the amount of iodine adsorbed was less than $12{\cdot}0 \times 10^{-7}$ moles I_2/mg. polysaccharide. In the ultraviolet there was a gradual fall in the levels of the absorption peaks, especially at the lowest concentration of iodine when the 287 mμ. peak was lower than the 353 mμ. peak. This is in agreement with Bebbington *et al.* (1952).

Assuming arbitrary values for the amount of molecular iodine taken up by the amylose from $6{\cdot}0 \times 10^{-7}$ moles/mg. to $12{\cdot}0 \times 10^{-7}$ moles/mg., the residual concentrations of free iodine in 0·001 M-KI were calculated. The appropriate absorption values for wavelengths 287 and 353 mμ. were intrapolated from the calibration curves for iodine solutions and subtracted from the observed value, at the same wavelength, of the amylose–iodine complex absorption curves. Since the polysaccharide was apparently saturated at all the iodine concentrations the difference at any wavelength should be constant when the correct iodine adsorption has been applied. The best fit lies about $7{\cdot}7$–$8{\cdot}0 \times 10^{-7}$ moles/mg. and the value 8×10^{-7} moles/mg. has been assumed with regard to the experimental errors involved. Complete light-absorption curves for the residual concentration of free iodine were then calculated based on this value for the iodine adsorption and subtracted from the observed curve in the presence of polysaccharide to determine the actual contribution from the amylose–iodine–iodide complex itself. It will be seen that reasonably good agreement is obtained over the range of iodine concentration used (Fig. 3).

Very similar conclusions were reached at the higher amylose concentration. From the visible absorption curves there was little doubt that saturation conditions were not reached below $7{\cdot}88 \times 10^{-5}$ M-I_2, i.e. the iodine adsorption lay between $10{\cdot}0 \times 10^{-7}$ and $8{\cdot}0 \times 10^{-7}$ moles/mg. The best statistical fit was obtained for an iodine adsorption of 9×10^{-7} moles/mg. and the contribution of the amylose–iodine–iodide complex was calculated as before using this value.

In these absorption curves (Fig. 3) there is a very slight change in gradient only in the region 287 mμ. but a pronounced peak at 350 mμ. followed by a minimum at 400 mμ. before rising to the peak in the visible. The extinction is proportional to the concentration of the amylose, which leaves little doubt as to the complete formation of the complex with iodine. The appreciably increased absorption for wavelengths shorter than 300 mμ. due to light scattering and a small superimposed peak at 250 mμ.

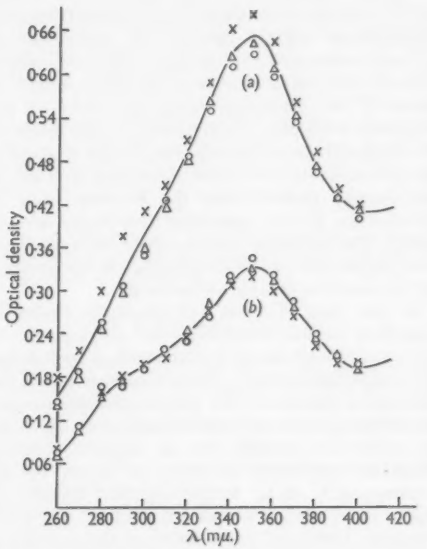

Fig. 3. Ultraviolet absorption curve of amylose–iodine–iodide complex in 0·001 M-KI. (*a*) Amylose concentration, 2 mg./25 ml.: ○, $12{\cdot}60 \times 10^{-5}$ M-I_2; △, $9{\cdot}46 \times 10^{-5}$ M-I_2; ×, $7{\cdot}88 \times 10^{-5}$ M-I_2 (iodine adsorption = $9{\cdot}0 \times 10^{-7}$ moles/mg.). (*b*) Amylose concentration, 1 mg./25 ml.; ○, $9{\cdot}46 \times 10^{-5}$ M-I_2; △, $6{\cdot}30 \times 10^{-5}$ M-I_2; ×, $4{\cdot}73 \times 10^{-5}$ M-I_2 (iodine adsorption = $8{\cdot}0 \times 10^{-7}$ moles/mg.).

(Laurent & Wertheim, 1952) have been neglected but would have the general effect of lowering the apparent absorption on the short-wavelength side of the absorption peak. The amounts of iodine adsorbed are higher than the value $7{\cdot}3 \times 10^{-7}$ moles/mg. quoted in the literature (Gilbert & Marriott, 1948; Bates *et al.* 1943) but can be due to the higher values obtained by the spectrophotometric method as already discussed above (Baldwin *et al.* 1944). This is in agreement with the higher value obtained at a higher concentration of amylose.

DISCUSSION

When iodine molecules are linked by simple covalent bonding (I–I = 2·67 Å) in the gaseous state and also in non-polar solvents (CS_2, CCl_4), a well-defined absorption band occurs at 520 mμ. (Liebhafsky, 1939). In the presence of electron-donor substances I–I is polarized to I^+–I^- or I^-–I^+ (Fairbrother, 1947, 1948). The contribution of these forms to the actual structure present determines the nature of the iodine spectrum. The potential energy above the ground state of the *p*, *s*, valency electrons, which influences light absorption, is increased in the polarized form and shifts the absorption maximum to shorter wavelengths, e.g. 460 mμ. in water, 462 mμ. in ethyl iodide, 490 mμ. in *n*-butyl chloride. Schlamowitz (1951) explains the movement of maximum absorption in amylose–iodine complexes towards the red with increasing chain length in terms of the gradually increasing effect of the supposed non-polar environment of the inside of the amylose helix on the adsorbed iodine molecules, but it is difficult to see how absorption maxima at wavelengths greater than the limiting value of 520 mμ. set by the unpolarized molecule are obtained. He concludes that the absorption spectrum of a polysaccharide–iodine complex is essentially a spectrum of the free iodine molecule.

In the case of short polyglucoside chains or branched polysaccharides of the amylopectin or glycogen type where the iodine bonding is weak and the hydrophobic region of the core very small, the absorption spectra of the polysaccharide complex would be expected to tend towards those exhibited by iodine or triiodide ion in aqueous solution. Essential consideration must be given to the presence of I^- or I_3^- in the adsorbed complex as indicated by the potentiometric titration (Gilbert & Marriott, 1948) and the mobility of the complex in an electric field (Mould & Synge, 1954). A linear configuration for I_3^- as a trigonal pyramid with the two external iodine atoms at the two pyramidal apices and three unbonded electron pairs in the equatorial plane around the central iodine atom has been established (Wyckoff, 1920; Mooney, 1935, 1937). The interaction of the I^- ion with a highly polarizable I_2 molecule leads to a small contribution of covalent bonding between the I^- and the nearer iodine of the I_2 group, giving the resonating structure

$$\overbrace{I^- \cdots I\text{–}I}^{3{\cdot}10\text{ Å}\quad 2{\cdot}82\text{ Å}} \qquad \overbrace{I\text{–}I \cdots I^-}^{2{\cdot}82\text{ Å}\quad 3{\cdot}10\text{ Å}}$$

Two absorption bands always appear, e.g. 287, 353 mμ., whenever this configuration is found (Allsopp, 1937; Potterill & Walker, 1937).

Stein & Rundle (1948) have accounted for the stability of the complex increasing with length of helix, in terms of the action of the dipole field of the helical polymer acting along the axis on a highly polarized array of halogen molecules. The field tends to reduce the van der Waals distance between neighbouring iodine molecules, and resonance occurs between the bonded and non-bonded iodine atoms so that the modified van der Waals and bonded distances tend to approach each other. A periodicity along the chain of 6·2 Å ($2 \times 3{\cdot}1$ Å) as found by West (1947) for other polymer–iodine complexes is not impossible according to this theory. Their calculations are, however, only strictly applicable for the case of adsorption of iodine vapour by solid dry amylose. Hach & Rundle (1951), following on studies of the I_5^- structure, have postulated that the distances apart of the iodine and iodide atoms in a linear resonating array can be between 3·0 Å and 3·1 Å. Cramer (1951) has reported a periodic distance of 3·06 Å in precipitated iodine–iodide complexes of Schardinger dextrins.

It appears likely that the suppression of the 287 mμ. peak in the amylose–iodine–iodide complex may be the result of change in electron orbital configuration due to the modified bond distance and certainly shows the absence of individual I_3^- structure. The resonance-energy contribution lowers the energy levels of the valency electrons and may be sufficient to shift the absorption maximum towards the longer wavelengths in the visible spectrum. It is suggested that with decreasing length of the helix the stable resonating extended chain breaks up into discrete complex groups and gradually increasing polarization of the I_2 molecules by the I^- ions now assumes a predominant role. This would have the effect of shifting the absorption maxima to shorter wavelengths and it would also be expected that the 287 mμ. peak would again show up as the I_3^- type structure gradually reappeared.

A spectrophotometric investigation of the red-staining polysaccharide (DP 34) similar to that carried out with amylose was inconclusive and an accurate absorption curve for the complex was not obtained. This was due to extensive surface-adsorbed or loosely bound iodine at the high free-

iodine concentration, also evident in the potentiometric titrations. It does not contribute to the absorption band in the visible but does contribute to the bands in the ultraviolet. The much smaller amount of iodine taken up in complex formation gave a small contribution to the visible absorption with a peak at about 510–520 mμ. The agreement of this value with that arising from covalent-bonded iodine is fortuitous as with still shorter chain lengths this value progressively falls to 490 mμ. (Whelan & Bailey, 1954). From arbitrary assumptions as to the amount of iodine adsorbed in the true helical complex taken from the potentiometric curves it would appear that the complex has a pronounced double peak in the ultraviolet (Fig. 4). This was borne out by changes in the maxima of the peaks when the polysaccharide was added in increasing amounts to iodine solution of constant concentration. The height of the 353 mμ. peak first fell and then rose with increased polysaccharide concentration. Observation of the corresponding changes in the 287 mμ. peak was not made, suitable light-absorption cells of small cross-section not being available. The effect may readily be explained by the induced formation of I_3^- type structures within the short helices of the polysaccharide increasing the proportion of the I_3^- ion in the polysaccharide–iodine–iodide solution more than would be expected under normal dissociation-equilibrium conditions. The field of the helix is presumably not long enough to stabilize a long chain and the interaction of the complex ions with each other are not enough to modify the I_3^- structure to any extent.

Cramer & Herbst (1952) have recently considered the formation of the absorption maxima exhibited by polysaccharide–iodine complexes in terms of the behaviour of a resonating iodine chain acting as a 'one-dimensional metal' (Kuhn, 1949). The calculated absorption maxima are of the right order of wavelength but decrease too rapidly with decrease in length of the iodine–iodide chain. This also may indicate that the short complexes cannot be considered as stable extended resonating chains.

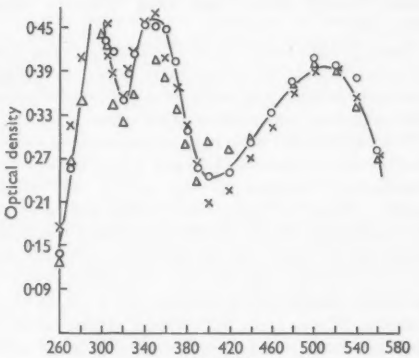

Fig. 4. Absorption curve of red-staining dextrin, DP 25–40, in 0·001 M-KI. Concentration of polysaccharide, 1 mg./25 ml.: ○, $25{\cdot}20 \times 10^{-5}$ M-I_2; △, $22{\cdot}08 \times 10^{-5}$ M-I_2; ×, $18{\cdot}92 \times 10^{-5}$ M-I_2. Iodine adsorption estimated from Fig. 1.

SUMMARY

1. The potentiometric titration with iodine of electrophoretically fractionated dextrins from α-amylase-hydrolysed amylose is described. The complex $3I_2$–yI^- is formed by the blue-staining fraction, degree of polymerization (DP) 40–90, and the complex $2I_2$–$y'I^-$ by the red-staining fraction, DP 25–40.

2. Experimental difficulties due to the high free-iodine concentration required for complex formation are discussed.

3. The ultraviolet absorption spectrum of the amylose–iodine–iodide complex has been determined and changes in the absorption spectrum given by the complexes formed by dextrins of shorter chain length are discussed.

4. It is suggested that with amylose the iodine–iodide chains form a stable resonating extended chain, but with decrease in length of helix this breaks up into discrete complex groups and the gradually increasing polarization of the I_2 molecules by I^- ions has the effect of shifting the absorption maxima to shorter wavelengths. From spectrophotometric data the presence of an I_3^- structure in the red-staining complexes is possible.

I am grateful to Dr G. A. Gilbert for helpful discussion and for the use of his potentiometric-titration apparatus at the University of Birmingham.

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Route of Absorption and Distribution of Oleic Acid and Triolein in the Rat

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(*Received* 26 *May* 1954)

During the last few years a better understanding of the process of intestinal fat absorption has been gained, largely with the aid of isotopically labelled material, in combination with the technique of Bollman, Cain & Grindlay (1948) for collecting intestinal or thoracic lymph from unanaesthetized rats. The results of these investigations have shown that after absorption long-chain saturated fatty acids are mainly transported via the intestinal lymph as triglycerides both in rats and cats (Bloom, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Bergström, Borgström, Carlsten & Rottenberg, 1950; Bloom, Chaikoff, Reinhardt & Dauben, 1951; Chaikoff, Bloom, Stevens, Reinhardt & Dauben, 1951; Bloom, Chaikoff & Reinhardt, 1951; Borgström, 1951, 1952*a*, *b*, *c*; Bergström, Borgström & Carlsten, 1954). Fatty acids with 10 or less carbon atoms are, however, mainly transported from the intestine via the portal blood vessels (Bloom, Chaikoff & Reinhardt, 1951; Kiyasu, Bloom & Chaikoff, 1952) largely in free form (Borgström, to be published). For summaries see Bergström & Borgström (1953, 1954).

The higher saturated fatty acids are thus not absorbed via different routes according to the state in which they enter the intestinal mucosa, i.e. as free acids or esterified with glycerol, as postulated in the original fat–fatty acid partition theory of Frazer (Frazer, 1938, 1943, 1946, 1948*a*, *b*, 1950, 1951, 1952).

There is also much experimental evidence that the unsaturated long-chain fatty acids are mainly absorbed via the lymphatics irrespective of whether they are fed as free acids or glycerides.

Munk (1880) fed oleic acid to dogs with a cannulated thoracic duct and found that there was a considerable increase in the neutral fat of the lymph. Munk & Rosenstein (1891) fed olive oil, oleic and erucic acid to a patient having a lymphatic fistula and obtained a high recovery of the ingested fat in the lymph. Freeman & Ivy (1935) obtained the same results after feeding olive oil to dogs. Bollman, Flock, Cain & Grindlay (1950) observed a marked increase of glycerides in the lymph after feeding oleic acid to dogs. Reiser & Bryson (1951) and Reiser, Bryson, Carr & Kuiken (1952) fed linoleic acid with conjugated double bonds to rats having a thoracic duct fistula and recovered part of the labelled acid in the lymph fat both after free acid and glyceride ingestion.

On the other hand, Frazer (1943), after feeding olive oil and oleic acid to rats and assessing the results with a technique mainly based on chylomicron counts and histological observations, concluded that 'fatty acid passes by the portal vein to the liver while neutral fat goes by the lymphatic route...' and this opinion has been reiterated in many subsequent publications (see above). Tidwell (1950), however, obtained a similar chylomicrographic response after feeding, respectively, olive oil and oleic acid to rats.

We now wish to report the results of some experiments in which [1-^{14}C]oleic acid has been fed either in free form or as triolein to rats with a thoracic-duct fistula. Some observations on the distribution of the labelled acid in these two forms to rats without a fistula have also been made.

EXPERIMENTAL

Methyl [1-^{14}C]oleate and [1-^{14}C]oleic acid were prepared according to Bergström, Pääbo & Rottenberg (1952), and triolein was prepared from oleic acid via the acid chloride prepared with oxalyl chloride.

The labelled substances were fed to rats with a thoracic-duct fistula (*A*) and also to animals without a fistula (*B*). The specific activity of the [1-^{14}C]oleic acid was 2300 counts/min./mg. (10 $\mu\mu$c/mg.).

A. *Animals with a thoracic-duct fistula*

Male rats weighing about 250 g. and starved for 12 hr. before operation were used. The procedure used for cannulating the thoracic duct was, with a slight modification, that described by Bollman *et al.* (1948). Instead of a straight cannula, a heated polythene cannula was bent at right angles about 0·5 cm. from the tip and was inserted through the left lateral body wall into the thoracic duct. This bend permits a cannula of larger calibre to be used, as the tension on the tube after insertion is reduced. After operation the rats were placed in restraining cages and had free access to 1% (w/v) NaCl solution until an adequate lymph flow was obtained. Thereafter, 0·64% (w/v) NaCl solution with 0·04% (w/v) KCl was fed *ad lib.* during the whole experiment. Three hours before the labelled substance was administered the rat had free access to white, fat-free bread. These rats were given either 0·5 ml. of labelled oleic acid or triolein by stomach tube when under light ether anaesthesia.

The lymph was collected for 24 hr., after which the animals were killed. In most of these experiments the rat in its cage was placed in a metabolic chamber and the expired CO_2 was recovered in NaOH. The lymph fat was extracted with ethanol–ether (3:1, v/v) and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)–$CHCl_3$ (2:1, v/v). The total fat from the lymph samples was separated into neutral fat and phospholipids on columns of silicic acid and these fractions were saponified as described by Borgström (1952*c*).

The whole gastrointestinal tract was removed in one piece and digested, together with the faeces collected during the experiment, with a solution of 30% (w/v) KOH at 100° for 12 hr. The mixture was then acidified and the fatty acids were extracted with light petroleum (b.p. 40–60°). The amount of fat absorbed in these experiments was calculated by taking the difference between the amount of isotope administered and the amount recovered from the intestinal tract and faeces.

The small activity present in the intestinal wall has thus been included in the unabsorbed amount in these cases, but this amount is negligible in fistula animals 24 hr. after feeding.

B. *Non-cannulated rats*

The labelled material was administered by gastric intubation in amounts corresponding to 0·1 ml./sq.dm. body surface (Lee, 1929). For comparison with the cannulated animals the activity of the expired CO_2 was measured on some rats which had received 0·5 ml. labelled material through a stomach tube. At different intervals after the feeding the rats were bled to death under ether anaesthesia. The gastrointestinal tract was removed and the contents of the small intestine were washed out with saline.

The small intestine was finely dispersed in ethanol in a Waring Blendor and the total fat extracted with ethanol–ether (3:1, v/v), and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)–$CHCl_3$ (2:1, v/v). The total fat was then fractionated into neutral fat and phospholipids on silicic acid columns and saponified (Borgström, 1952*c*).

Measurement of radioactivity

The determination of ^{14}C in fatty acids was done with a thin end-window Geiger-Müller tube on samples of 'infinite thinness'. Samples of an ethanolic solution were evaporated on aluminium planchets to leave less than 0·2 mg./cm^2.

In order to relate the activity of the fatty acids and the activity of the expired CO_2 that was counted as $BaCO_3$ at 'infinite thickness', suitably diluted samples were plated as described above and similar samples were oxidized to CO_2 and counted as $BaCO_3$ at 'infinite thickness' (Bergström, Borgström & Rottenberg, 1951).

RESULTS

The results of the lymph experiments (*A*) are given in Table 1. When labelled oleic acid was fed as the free acid, 66–92% (mean 78·2%) of the fed activity was absorbed in 24 hr. Rats receiving labelled triolein absorbed 57–92% (mean 76·2%) of the activity. It is seen that the fed free oleic acid was absorbed just as well as triolein in 24 hr. After feeding free [1-^{14}C]oleic acid, 38–96% of the absorbed activity was recovered in the lymph fat from the thoracic duct (mean 63·0%). The corresponding figures obtained when labelled triolein was fed were 51–83% (mean 65·5%). Feeding the labelled oleic acid as the free acid or as the glyceride made no significant difference to the recovery of the activity in the lymph lipids.

About 2% of the activity found in the lymph lipids was in the form of phospholipids, the remainder in the form of neutral fat in both groups. It was found that the labelled fatty acid in the lymph lipids followed the mono-unsaturated fatty acid within the limits of the fractionation method of

Table 1. *Recovery of* [^{14}C]*oleic acid and* [^{14}C]*triolein in thoracic lymph after oral administration to rats*

Rat no.	Substance fed	Amount of administered activity absorbed (%)	Amount of absorbed activity recovered in lymph as fatty acids (%)	Amount of lymph fat activity found in: Glyceride fatty acids (%)	Amount of lymph fat activity found in: Phospholipid fatty acids (%)	Amount of absorbed activity in expired CO_2 (%)
1	Oleic acid	91·5	64·0	98·2	1·8	4·8
2	Oleic acid	65·5	38·1	98·8	1·2	3·7
3	Oleic acid	73·0	96·4	97·4	2·6	3·5
4	Oleic acid	91·8	62·5	97·9	2·1	—
5	Oleic acid	69·0	53·8	97·5	2·5	4·5
Mean		**78·2**	**63·0**	—	—	—
6	Triolein	92·1	59·3	97·5	2·5	4·4
7	Triolein	56·5	50·5	97·7	2·3	5·0
8	Triolein	85·0	69·2	97·4	2·6	4·0
9	Triolein	71·0	82·9	98·8	1·2	—
Mean		**76·2**	**65·5**	—	—	—

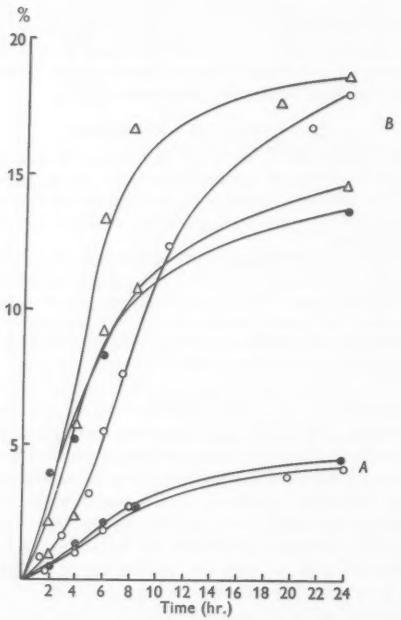

Fig. 1. Percentage of absorbed isotope in expired CO_2 after feeding [^{14}C]oleic acid as triolein (●), methyl oleate (△) or free oleic acid (○). *A*, rats with lymph fistula; every point represents the mean of three animals. *B*, rats without fistula; each point represents a single animal.

Bergström & Pääbo (1954). This method is based on chromatographic separation of the products of hydroxylation of the mixture of saturated and unsaturated fatty acid with performic acid.

While non-cannulated rats fed [1-^{14}C]oleic acid or methyl [1-^{14}C]oleate expired about 16% of the absorbed activity as carbon dioxide within 24 hr., rats with the thoracic duct cannulated expired in the same time about 4% irrespective of whether the oleic acid had been administered as glyceride or as free acid (Fig. 1).

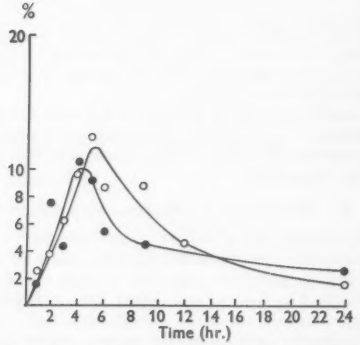

Fig. 2. Relative specific activity of phospholipid fatty acids of small intestine after feeding [^{14}C]oleic acid (○), or [^{14}C]triolein (●).

In Fig. 2 are given the curves for the specific activities of the fatty acids of the phospholipids of the intestinal wall as percentage of the specific activity of the fed fatty acids. The specific activities of the phospholipid fatty acids increased to a maximum of about 10% of that of the fed fatty acids about 4 hr. after administration. However, only a part of these phospholipids represents material that had been present in the epithelial cells of the mucosa.

DISCUSSION

After feeding labelled free oleic acid or triolein to rats with the thoracic duct cannulated, means of 63·0 and 65·5% of the absorbed activity were

recovered in the lymph lipids during the following 24 hr.

These figures give for the first time quantitative information about the route of transport of oleic acid after absorption from the small intestine.

In corresponding experiments with [^{14}C]palmitic acid dissolved in olive oil (Blomstrand, 1954), in which the same procedures were used as in the experiments reported here, a mean of 67·4 % of the absorbed activity was recovered in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–75·0 in five experiments). If these figures obtained for labelled palmitic acid are compared with those obtained for oleic acid in this investigation no significant differences are found.

The figures obtained for palmitic acid recovered in the lymph in these experiments are not as high as those given by Bloom *et al.* (1950). These authors found a mean of 75·9 % of the absorbed activity in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–91·8 in ten experiments). The differences of the means in these two series of experiments may be due to differences in the analytical methods used. Bloom *et al.* (1950) determined the radioactivity directly from the total fatty acids of the lymph obtained after saponification, while in experiments from this laboratory the neutral and phospholipid fatty acids were isolated by a chromatographic procedure before the activity determinations. In this way small losses of activity probably occurred.

Probably there are several causes for the large variation between different animals. There are certainly considerable anatomical differences between different animals in the arrangement of lymph vessels, some of which might by-pass the fistula. Possibly anastomoses exist between lymph and blood vessels which make possible an increased by-passing of the thoracic duct if the lymph flow is partly blocked by, for instance, clots that form in the lymph vessels of some cannulated animals. Also the lymph flow is probably not entirely normal with an open fistula. It is not unlikely that the rats with the highest recoveries are those most approaching normal.

It is seen in Fig. 1, however, that the amount of isotope expired during 24 hr. by a cannulated rat is roughly one-fourth to one-third of the amount expired by the non-cannulated rat. This corresponds roughly to the loss of labelled material via the fistula and shows that the absorbed labelled compounds are metabolized at a normal rate. It must be emphasized that the extent of lymphatic transport of oleic acid was found to be the same as palmitic acid when studied with the same technique (Blomstrand, 1954). No difference was observed in the recovery of labelled oleic acid in the thoracic-duct lymph lipids during the 24 hr. following the administration of labelled oleic acid as free acid or as triolein. The capacity of the intestinal cells to synthesize triglycerides is thus apparently very great. These findings are in general in agreement with the results earlier obtained on the absorption of long-chain unsaturated fatty acids in humans, dogs and rats by Munk (1880), Munk & Rosenstein (1891), Freeman & Ivy (1935), Bollman *et al.* (1950) and Fernandes (1953).

Frazer (1943) followed the absorption of oleic acid and olive oil in the rat with chylomicron counting and reached other conclusions. In the case of olive oil he observed a quicker and stronger response, which led him to the interpretation that the free oleic acid fed was absorbed directly via the portal route and rapidly metabolized in the liver, whereas the olive oil was mainly absorbed via the lymphatics and deposited in the fat depot. These interpretations formed part of the basis for the original 'fat–fatty acid partition theory'. Chylomicron counting—a method of doubtful quantitative significance—seems unsuitable for studies of this kind and has led to erroneous interpretations of this phase of the absorption process (for further discussion cf. Bergström & Borgström, 1954).

That fatty acids of different structure are incorporated into the phospholipids of the small intestinal wall to different extents has been found in earlier work with elaidic acid (Sinclair & Smith, 1937), conjugated linoleic acid (Barnes, Miller & Burr, 1941), and with different saturated fatty acids (Bergström *et al.* 1952; Borgström, 1952*d*).

The values obtained for the incorporation of labelled oleic acid in this work correspond to those found with double-bond conjugated linoleic and myristic acids.

In conclusion it must be emphasized that a very great variability in the rate of the absorption of fat and fatty acids is to be expected. Not only is the general condition of the animal of importance but the presence and nature of other food constituents greatly affect the absorption rate. The influence of these factors on the route of absorption and distribution in the body seems to be of less importance.

SUMMARY

1. The intestinal absorption of [1-^{14}C]oleic acid has been studied in rats with cannulated thoracic ducts.

2. It was found that oleic acid was transported via the thoracic-duct lymph and incorporated into triglycerides and phospholipids to the same extent as found for palmitic acid in earlier work. The same results were obtained whether the oleic acid was fed as free acid or as triolein.

3. The incorporation of the labelled acid into the lipids of the intestinal wall and the appearance of the isotope in the expired carbon dioxide has been followed.

4. The results indicate clearly that there is no detectable difference between the routes followed during absorption of fed free oleic or palmitic acids, or of glycerides containing these acids.

The technical assistance of Miss M. B. Tegman is gratefully acknowledged. This work is part of investigations supported by 'Statens Medicinska Forskningsråd' and 'Knut och Alice Wallenbergs Stiftelse'.

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Intestinal Absorption and Metabolism of 2:2-Dimethylstearic Acid in the Rat

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(*Received* 26 *May* 1954)

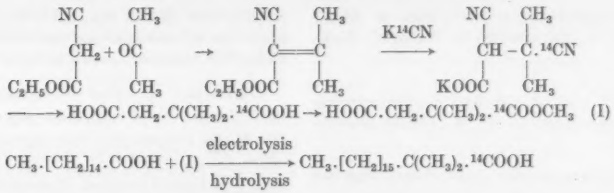
The intestinal absorption of different straight-chain carboxylic acids has been extensively studied during recent years (for a summary see Bergström & Borgström, 1954).

In order to investigate the influence of steric hindrance at the carboxyl group we have prepared [*carboxy*-^{14}C]2:2-dimethylstearic acid and studied its absorption and metabolism in the rat.

EXPERIMENTAL AND RESULTS

A survey of the methods previously used for the preparation of 2:2-dimethylstearic acid is given by Cason, Sumrell & Mitchell (1950). In the present work it has been prepared by the following reactions.

2:2-Dimethylsuccinic acid. 2:2-Dimethylsuccinic acid was prepared by a slight modification of the method of Vogel (1928). Solutions of ethyl 2-cyano-3:3-dimethylacrylate (20 g.) in ethanol (100 ml.) and KCN (11 g.) in water (25 ml.) were mixed and kept at room temperature for 24 hr. The ethanol was removed by distillation. After cooling, a very large excess of concentrated HCl was added and the solution refluxed for 3 hr. After dilution with water and addition of $(NH_4)_2SO_4$, it was extracted five times with ether. The ether solution was dried with Na_2SO_4 and evaporated to dryness. Impurities were removed by boiling the product twice with small amounts of benzene, cooling to room temperature and collecting the crystals by filtration. Yield 13·2 g. (69%); m.p. 142°. (Found: equiv. 73·1. Calc. for $C_6H_{10}O_4$, 73·1.)



2:2-Dimethyl [1-^{14}C]succinic acid was prepared analogously with [^{14}C]cyanide. One-twentieth of the above quantities was used, except that the ethyl 2-cyano-3:3-dimethylacrylate was here in slight excess. The yield was diminished to 63%, and the melting point of the labelled product was 143°.

Methyl hydrogen 2:2-dimethylsuccinate (3-methoxycarbonyl-3-methylbutyric acid) (cf. Bone, Sudborough & Sprankling, 1904). 2:2-Dimethylsuccinic acid (11·0 g.) was dissolved in methanol and treated with diazomethane in ether solution. The solvents were removed by distillation. The diester formed was converted into the half-ester

$$HOOC.CH_2.C(CH_3)_2.COOCH_3$$

by hydrolysis with 1·67 N methanolic KOH solution (45·1 ml.) and water (5 ml.). The alkaline solution was kept at room temperature overnight and was then refluxed for 3–4 hr. After addition of water and acidification with HCl, the ester was extracted with ether. The ether solution was dried with Na_2SO_4. After evaporation of the ether the product was dried *in vacuo*. The yield of crude product was 11·5 g.

Methyl hydrogen 2:2-dimethyl [1-^{14}C]succinate was prepared analogously.

2:2-Dimethylstearic acid. Methyl hydrogen 2:2-dimethylsuccinate (3-carbomethoxy-3-methylbutyric acid) (5·7 g. of crude product), palmitic acid (20 g.) and sodium (0·45 g.) were dissolved in methanol (300 ml.) and electrolysed (Pt electrodes) with a current density of about 0·3 A/cm.^{2} (cf. Ställberg-Stenhagen, 1950, 1951). During the electrolysis external cooling with ice was necessary and also addition of methanol to replace loss due to evaporation. When the reaction was alkaline to litmus, another 5·7 g. of methyl ester and 20 g. of palmitic acid were added, and the electrolysis was continued until the reaction was slightly alkaline. After cooling to room temperature, the triacontane which had precipitated was filtered off, extracted once with boiling methanol and again filtered after cooling.

The combined methanol solutions were evaporated to dryness and the solid residue was extracted with ether 3 times. The excess palmitic acid was removed by washing the ether solution with portions of Na_2CO_3 solution until no more palmitate precipitated. The precipitate was removed by filtration, and the solution was washed with water until the reaction was neutral. After evaporation of the ether the product was hydrolysed by heating it with KOH (25 g.), water (10 ml.) and ethanol (170 ml.) for 6 hr. at 120° in an autoclave. The hydrolysate was diluted with water (150 ml.) and extracted twice with petroleum ether. The water–ethanol layer was acidified with dil. HCl and extracted with ether. The ether solution was dried with Na_2SO_4. Evaporation to dryness yielded 2 g. of crude acid. Another 7 g. of acid were obtained by extraction of the light petroleum solution with 50% (w/v) ethanol, acidification of the ethanol–water solution and extraction with ether. After crystallization from methanol of the combined acid products, 6·8 g. of 2:2-dimethylstearic acid were obtained (29% of the theoretical amount, calculated on 2:2-dimethylsuccinic acid) m.p. 57°. After recrystallization from ligroin the m.p. was 58·5°. (Found: C, 76·8; H, 12·8. Calc. for $C_{20}H_{40}O_2$: C, 76·9; H, 12·9.)

2:2-Dimethyl [1-^{14}C]stearic acid was prepared analogously on a small scale.

Preparation of 2:2-dimethyladipic acid. Sodium (0·4 g.) was dissolved in methanol (300 ml.), and methyl hydrogen 2:2-dimethylsuccinate (3·6 g., 0·0225 mole) and methyl hydrogen succinate (12 g., 0·09 mole) were added. The electrolysis was carried out as described above. The reaction mixture was taken to dryness and extracted with several portions of boiling ether.

The combined ether extract was washed with a 5% (w/v) solution of Na_2CO_3 and with water. The ether was distilled off and the residue (5·7 g.) was partially hydrolysed by means of 1·67 N methanolic KOH (82 ml.) and water (4·5 ml.) at room temperature overnight. After hydrolysis, water (100 ml.) was added and the alkaline solution was extracted twice with ether to remove methyl tetramethyladipate. The solution was acidified with HCl and extracted 5 times with ether. The combined ether fractions were then dried with Na_2SO_4 and the ether was removed. The residue (4·3 g.) was partitioned between equal volumes (80 ml.) of $CHCl_3$ and water. The $CHCl_3$ phase was evaporated to dryness and the residue (1·2 g.) was hydrolysed in an autoclave at 110° for 5 hr. with KOH (5 g.) in water (2 ml.) and ethanol (45 ml.). After saponification the solution was acidified and extracted 3 times with ether. Crystallization of the crude acid from water yielded 520 mg. of 2:2-dimethyladipic acid, m.p. 81°. (Found: C, 55·1; H, 8·1. Calc. for $C_8H_{14}O_4$: C, 55·2; H, 8·1.)

Administration of 2:2-dimethyl [1-^{14}C]stearic acid to rats. The animals used were adult white male rats weighing about 250 g. and were kept in metabolism cages during the experiments. White bread and water were fed *ad lib.* For further experimental details see the preceding paper (Bergström, Blomstrand & Borgström, 1954). For these experiments a stock solution of 103·5 mg. of 2:2-dimethyl [1-^{14}C]stearic acid in 5·1 g. of olive oil was used.

Measurement of radioactivity

The isotope content of the synthetic compounds was determined with thin end-window Geiger-Müller tubes after evaporating samples of an ethanolic solution on aluminum planchets to leave less than 0·2 mg./cm.^{2} The urine and faecal extracts were also counted after evaporation on planchets. The self absorption was determined by evaporating samples to which known amounts of highly active dimethyladipic or dimethylstearic acid respectively

had been added. Expired CO_2 was absorbed in alkali, precipitated as $BaCO_3$ and counted in 'infinite' thickness.

A. *Recovery of* [^{14}C] *in excreta and expired carbon dioxide after administration of* [^{14}C]*dimethylstearic acid*

A rat was fed 0·65 ml. of the stock solution (532 000 counts/min.) by gastric intubation under light ether anaesthesia. It was placed in a 4 l. desiccator at 25–28° through which a constant stream of CO_2-free air was passed at a constant rate of about 500 ml./min. The CO_2 in the expired air of the rat was collected in NaOH solution and the amount of CO_2 determined by titration of a sample. To another sample NH_4Cl and $BaCl_2$ were added. The precipitate of $BaCO_3$ was collected and the isotope content determined on infinitely thick samples. However, the activity was so small that it could not be determined with this method.

The urine was collected on the bottom of the desiccator. From the first 24 hr. the total urine activity was 368 000 counts/min., i.e. 69 % of the amount administered. The urine from 24–72 hr. after administration contained 42 500 counts/min., i.e. 8 % of the given amount. Consequently, a total of 77 % was recovered in the urine.

Faeces were collected on a metal net in the desiccator. After collection, the faeces from the first day were digested in 30 % (w/v) KOH on a steam bath for 12 hr. After acidification with HCl they were extracted with ether.

The total activity was 48 000 counts/min., i.e. 9 % of the administered activity. During the second and the third day 5 and 3 % respectively was recovered, i.e. a total of 17 % was found in the faeces.

B. *Administration of* [^{14}C]*dimethylstearic acid to a rat with a lymph fistula*

A rat with a cannula in the thoracic duct was fed 0·5 ml. of the olive oil–dimethylstearic acid mixture (410 000 counts/min.) through a stomach tube. It was placed in a special cage similar to that described by Bollman, Cain & Grindlay (1948), and the lymph was collected for 24 hr. The total lymph fat was separated on a column of silicic acid (Borgström, 1952*a*). The lipids eluted with chloroform (416 mg.) contained a total of 147 000 counts/min. The phospholipids were then eluted with methanol and had a total activity of 5500 counts/min. (3·6 % of the total fat). The chloroform eluate was passed through the ion-exchanger Amberlite IRA-400 as described by Borgström (1952*b*) to ensure that no trace of free fatty acids was present. The neutral fat (390 mg.) had a total activity of 127 500 counts/min., i.e. 87 % of the total lymph-fat activity or 31 % of the total amount administered.

Hydrolysis of lymph fat with bile–pancreatic juice. A sample (120 mg.) of neutral fat from the above-mentioned fraction was hydrolysed with 5 ml. rat bile–pancreatic juice according to the procedure described by Borgström (1952*c*). The pH was adjusted to 6·3, the reaction temperature was 40° and analyses were made after 1, 3, 6, 12 and 24 hr. The results are shown in Table 1.

Hydrolysis of olive oil containing free [^{14}C]*dimethylstearic acid with bile–pancreatic juice.* When the above experiment was repeated with unlabelled olive oil in which 2 % of free [^{14}C]dimethylstearic acid had been dissolved, the results shown in Table 2 were obtained. The activity of the mixture was 900 counts/min./mg.

The data in the last column should be compared with corresponding experiments in which free labelled palmitic or oleic acid had been added (Borgström, 1954). In these experiments the glyceride fatty acids had reached about 40 % of the activity of the free fatty acids already after 2 hr. incubation.

C. *Identification of the main isotopic compound in urine after administration of* [^{14}C]*dimethylstearic acid*

In an experiment similar to *A*, 1 ml. of the solution (820 000 counts/min.) was fed to a rat. The urine was collected for 48 hr. after the administration. The activity was 517 000 counts/min., i.e. 63 % of the given amount. The acidified urine was extracted 3 times with 3 vol. of ether. The total activity of the ether phase was 350 000 counts/min. The total activity of the aqueous phase after ether extraction was 134 000 counts/min. Consequently, 72 % of the activity was in the ether phase.

Table 1. *Distribution of radioactivity during enzymic hydrolysis of lymph fat*

For details see text.

Time (hr.)	Amount of hydrolysis (%)	Radioactivity (counts/min./mg.)	
		Neutral fat	Acids
1	22·4	424	—
3	35·8	463	16
6	53·0	616	26
12	72·2	818	20
24	86·2	1502	30

Table 2. *Distribution of radioactivity during enzymic hydrolysis of lymph fat*

For details see text.

Time (hr.)	Amount of hydrolysis (%)	Radioactivity of glyceride fatty acids (counts/min./mg.)	Activity of glyceride fatty acids as a percentage of the activity of free fatty acids
1	18·2	3	0·2
2	32·6	9	0·9
4	43·4	12	1·1
6	52·8	16	1·8

CORRIGENDUM

Intestinal absorption and metabolism of 2:2-dimethylstearic acid in the rat

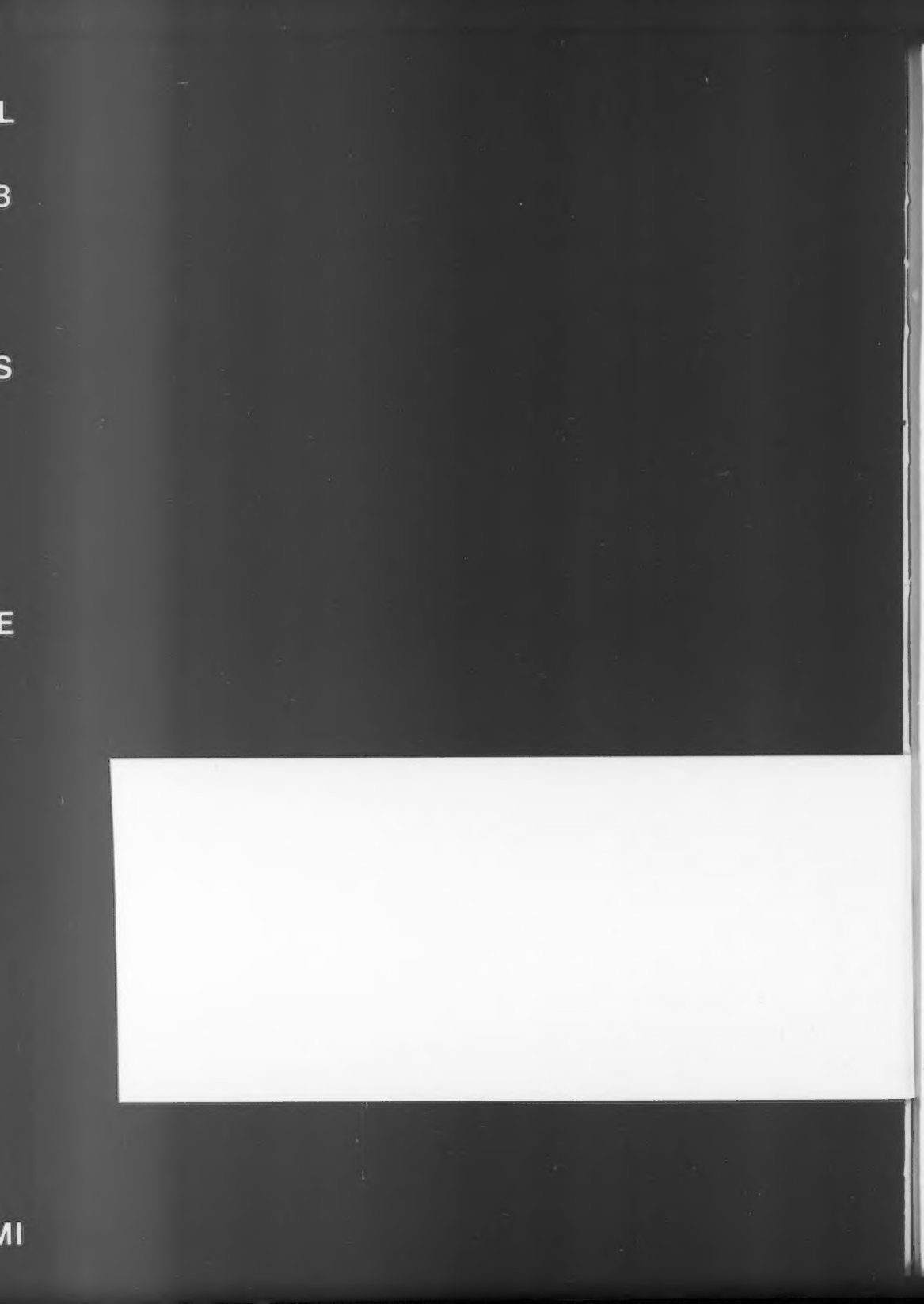
By S. Bergström, B. Borgström, N. Tryding and G. Westöö

Volume 58 (1954)

p. 606, Table 2, legend:

for *Distribution of radioactivity during enzymic hydrolysis of lymph fat*

read *Distribution of radioactivity during enzymic hydrolysis of olive oil containing free* [^{14}C]*dimethylstearic acid*



Hydrolysis of the aqueous phase. After alkaline hydrolysis 45% of the remaining activity could be extracted with 3 vol. of ether. This indicates that the urine compound was partly in conjugated form.

Chromatography of the ether extract of the urine. About 4% of the residue of this ether extract was mixed with 2:2-dimethylsuccinic acid (7·9 mg.) and 2:2-dimethyladipic acid (6·3 mg.) and subjected to reversed-phase partition chromatography according to the method of Howard & Martin (1950), with phases similar to those used in the separation of bile acids by Bergström & Sjövall (1951) and Norman (1953). Stationary phase: 50% (v/v) *iso*octanol–chloroform, moving phase: 40% (v/v) aqueous methanol. 4·5 g. of hydrophobic kieselguhr (Hyflo Supercel treated with silane) and 4 ml. stationary phase were used. The rate of flow was regulated to 2·5 ml./5 min. Each fraction was titrated with 0·02N-NaOH (bromthymol blue) and then evaporated to dryness in an oven at 105°. The contents of each tube were dissolved in 50% (v/v) ethanol and transferred to copper planchets. From some tubes samples were taken so that the weight of substance did not exceed 0·5 mg. on each planchet.

The results are shown in Fig. 1. Almost 90% of the activity was contained in the second band which corresponds to dimethyladipic acid.

Recrystallization of 97·8 mg. of 2:2-dimethyladipic acid and about 18% of the ether extract of the urine was performed with water and benzene–light petroleum (b.p. 40–60°) alternately. The specific activity of the crystals stayed constant during six recrystallizations from water and benzene–light petroleum.

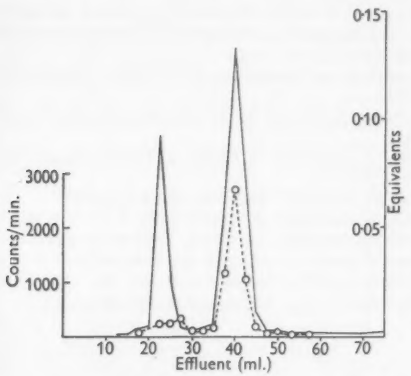


Fig. 1. Partition chromatography of urinary extract from experiment *C* together with 2:2-dimethylsuccinic acid (7·9 mg.) and 2:2-dimethyladipic acid (6·3 mg.); stationary phase: *iso*octanol–$CHCl_3$ (1:1, v/v) supported on 4·5 g. hydrophobic Supercel. Moving phase: 40% (v/v) aqueous methanol. Titration values, solid line; counts/min./fraction, dotted line.

DISCUSSION

The results presented indicate that 2:2-dimethylstearic acid was well absorbed from the intestine when fed in free form dissolved in olive oil. The extent of transport via the lymphatic pathway appears from the limited data to be approximately the same as for the ordinary long-chain fatty acids with the same technique and in the same strain of animals (cf. Bergström *et al.* 1954; Blomstrand, 1954). The labelled acid that had been administered in free form was transported via the lymphatics incorporated into glycerides and phospholipids as is the case with the ordinary straight-chain fatty acids.

However, the α-disubstituted acid showed a different behaviour with pancreatic lipase. When a labelled straight-chain fatty acid was present when glycerides were split by pancreatic lipase it was found by Borgström (1952*c*, 1954) that a rapid incorporation of the labelled fatty acid into glyceride ester bonds occurred simultaneously with the overall hydrolysis. This has been demonstrated *in vitro* as well as *in vivo* in the intestinal contents of rats. In this work it has been found that the 2:2-dimethyl-substituted acid only became incorporated in traces under comparable experimental condition. The reason might be ascribed to the steric influence of the substituents at the α-carbon atom that also causes the well-known effect on the rate of esterification and hydrolysis of acids and esters of this type. The substituents might possibly also hinder an appropriate close packing of the acid with the glycerides and straight-chain fatty acid at the interface where the lipase is acting.

Glyceride ester bonds of the branched chain acid were also found to be almost resistant to the action of pancreatic lipase. When the lymph triglycerides containing the labelled acid were subjected to the action of rat bile–pancreatic juice the usual rapid hydrolysis occurred. However, almost all of the labelled branched-chain fatty acid remained bound in the glyceride residue, indicating that a glyceride ester bond of the substituted acid was virtually resistent to the action of pancreatic lipase. It appears likely that the steric effect of the two methyl groups close to the ester bond hinders the appropriate contact between the lipase molecule and the substrate that is necessary for hydrolysis to occur.

The fact that the substituted acid had been incorporated into glyceride ester bonds during the passage through the intestinal cells shows that the synthesis of ester bonds which is taking place in the intestinal cells as expected is not a simple reversal of the lipase mechanism. Presumably this synthesis is taking place via acyl transfer from energy-rich S-acyl compounds, by analogy with the mechanism shown to occur in liver when phosphatidic acid is acylated (Kornberg & Pricer, 1953).

The results show that most of the absorbed dimethylstearic acid that had been transported via the lymphatics incorporated into neutral fat and phospholipids had thus been rapidly set free and metabolized in the body of the rat. The disubstitution at the α-carbon atom hinders the usual β-oxidation completely and the molecule is instead ω-oxidized. The dicarboxylic acid had then been β-oxidized from the unsubstituted end 6 times leaving the substituted adipic acid with six carbon atoms and this was rapidly excreted in the urine.

These results are in general accordance with earlier extensive work on the metabolism of fatty acids with branched chains. For a summary see Weitzel (1950, 1951). Kuhn, Köhler & Köhler (1936) found that α-methylstearic acid was completely metabolized in the rabbit without any products being found in the urine. As α-methyladipic acid itself when administered appears in the urine to 60–70 %, these experiments indicate that β-oxidation from the carboxyl end had occurred to a considerable extent. Thus, monosubstitution at the α-carbon atom does not appear to hinder β-oxidation but when the substituent becomes larger (ethyl < propyl < butyl) more acid degradation products are excreted with the urine mainly as the corresponding substituted adipic acids. After feeding α-*n*-butylstearic acid to dogs, about 6·5 moles per cent could be recovered in the urine as α-butyladipic acid (Weitzel, 1951).

The experiments recorded in this work show that two methyl groups at the α-carbon completely hinder any β-oxidation from this end of the molecule. The recovery of 90 % of the absorbed isotope as [^{14}C]dimethyladipic acid also shows that a rapid and complete ω-oxidation followed by β-oxidation had taken place.

SUMMARY

1. [*carboxy*-^{14}C]2:2-Dimethylstearic acid and 2:2-dimethyladipic acid have been synthesized.

2. The former acid was well absorbed when dissolved in olive oil and administered to rats.

3. The absorbed acid was transported via the lymphatics incorporated into glycerides and phospholipids.

4. No isotope was found in the expired carbon dioxide, but more than 90 % of the absorbed isotope was recovered in the urine as [^{14}C]dimethyladipic acid.

5. Dimethylstearic acid did not take part in the formation of ester bonds occurring during hydrolysis of olive oil with pancreatic lipase. When the lymph glyceride containing this acid was treated with bile–pancreatic juice, this ester bond appeared virtually resistant to the action of lipase.

6. Other types of enzymes which apparently are not hindered by the two α-methyl groups affect the synthesis of glyceride ester bonds in the intestinal cells and the splitting of these bonds in the tissues of the rat.

This work is part of investigations supported by 'Statens Medicinska Forskningsråd', 'Knut och Alice Wallenbergs Stiftelse' and 'Magn. Bergvalls Stiftelse'.

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A Glucuronide-decomposing Enzyme from Rumen Micro-organisms

2. PURIFICATION AND KINETICS

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(*Received* 25 *June* 1954)

The first paper in this series (Karunairatnam & Levvy, 1951) reported the discovery of an enzyme, normally present in micro-organisms in the rumen of the sheep, which decomposed β-D-glucosiduronic acids. Other enzymes, apparently having a similar action, have since been reported in the contents of the alimentary tracts of several species of mammals (Marsh, Alexander & Levvy, 1952), in the crop liquor of locusts (Robinson, Smith & Williams, 1953) and in the digestive tracts of some marine molluscs (Dodgson, Lewis & Spencer, 1953). These enzymes have provisionally been classified as β-glucuronidases, until their modes of action and specificities have been more thoroughly studied. A further examination of the enzyme occurring in the sheep rumen has been made, and in this paper a method of extraction and purification of the enzyme and the kinetics of enzyme action are described.

It has not yet been ascertained with which organism, or group of organisms, this enzyme is associated. The method of Karunairatnam & Levvy (1951), using as starting material a centrifuged fraction of the mixed rumen population, has therefore been adopted. These workers prepared cell-free extracts by mechanical disintegration of the organisms; for larger preparations it was found more convenient to prepare aqueous extracts of acetone-treated powders. The later stages of purification of the enzyme were controlled by the use of the specific-property solubility test of Falconer & Taylor (1946), who used it as a criterion of absolute purity of a liver esterase preparation. For the multi-stage purification of an enzyme by salt precipitation it has been found very convenient in defining at each stage the limits of salt saturation to be employed at the following stage, in order to achieve a known degree of purification with economical recovery of enzyme activity.

EXPERIMENTAL AND RESULTS

Materials and general methods

Substrates. Biosynthetic phenolphthalein β-D-glucuronide (Fishman, Springer & Brunetti, 1948), phenyl β-D-glucuronide (Garton, Robinson & Williams, 1949) and (−)-menthyl β-D-glucuronide (Williams, 1938) were used.

Measurement of enzyme activity. The liberation of phenolphthalein from phenolphthalein glucuronide (5×10^{-4} M) in 0·3 N-H_3PO_4 : NaOH buffer, in 1 hr. at 38°, was measured by the procedure of Karunairatnam & Levvy (1951); one unit of enzyme activity was defined as that which liberated 1 μg. phenolphthalein/hr. at pH 6·1 from this substrate. The relative purity of an enzyme preparation was measured in terms of its specific activity, i.e. activity units/mg. N.

In experiments using phenyl glucuronide as the substrate, the liberation of phenol by the enzyme in 0·05 N citric acid–NaOH buffer was measured by a modification (Levvy & Marsh, 1952) of the procedure of Kerr, Graham & Levvy (1948). Preliminary experiments showed that, as in the case of animal β-glucuronidase (Kerr *et al.* 1948), there was quantitative recovery of added phenol after incubation with the enzyme preparation.

Attempts to measure the enzymic liberation of reducing sugar from (−)-menthyl glucuronide in 0·05 N citric acid–NaOH or 0·2 N phthalic acid–NaOH buffers were made using the cerimetric method as described by Levvy (1946). The ceric sulphate solution (approx. 0·012 N) was standardized by arsenious oxide (A.R.) (Vogel, 1943). In the determination of the glucuronic acid equivalent of ceric sulphate according to the procedure of Levvy (1946) it was found that D-glucurone and D-glucuronic acid (prepared by titrating a D-glucurone solution with diluted alkali to a stable, slightly alkaline pH) had equivalent reducing powers. It was calculated that 1 μg. glucuronic acid was equivalent to 0·002 ml. 0·0127 N ceric sulphate; Levvy (1946) gives the equivalent of 1 μg. glucuronic acid = 0·002 ml. 0·0124 N ceric sulphate. Protein precipitation was carried out using copper tungstate (Levvy, 1946) or 5% (w/v) trichloroacetic acid (TCA) (Fishman, 1939*a*). The former method necessitated the use of phthalate buffer; when TCA was used the concentration of Na_2CO_3 in the alkaline ferricyanide was increased accordingly to neutralize the free acid present.

Nitrogen determinations. For stages before the preparation of cell-free extracts of the enzyme, determination of total N was performed. For cell-free extracts, protein N was determined after precipitation with 5% TCA. After standing at 0° for 12 hr., the precipitate was sedimented by centrifuging at 1500 *g* for 15 min. and washed thrice on the centrifuge with 5% TCA. Control experiments showed that this removed all traces of $(NH_4)_2SO_4$ introduced in previous operations. The precipitate was finally resuspended in water and samples removed for duplicate determinations of protein N.

Kjeldahl digestion and measurement of total or protein N were as described by Karunairatnam & Levvy (1951).

Ammonium sulphate precipitation. Since the solubility of $(NH_4)_2SO_4$ varies appreciably with the temperature, an aqueous solution (*ca.* 3·5 M) saturated at 0° was used for

protein precipitation. A calculated quantity was added to the enzyme solution, buffered with 0·3N-H_3PO_4–NaOH, pH 6·1, at 0°, to give the desired $(NH_4)_2SO_4$ concentration, expressed as percentage saturation. The slight error thereby introduced owing to the volume contraction on dilution of the saturated $(NH_4)_2SO_4$ solution was neglected. After precipitation, the mixture was allowed to equilibrate at 0° for 30 min. before centrifuging at 1500 *g* for 15 min. at 15°. The required precipitates after $(NH_4)_2SO_4$ fractionation were redissolved in water and dialysed against running tap water for 48 hr. before further fractionation or use of the specific-property solubility test.

Specific-property solubility tests. The enzyme solution, buffered with 0·3N-H_3PO_4–NaOH, pH 6·1, was added to suitable mixtures of saturated $(NH_4)_2SO_4$ and water to give systems of equal volumes and enzyme content with differing $(NH_4)_2SO_4$ concentrations. After standing at 0° for 30 min., and centrifuging at 1500 *g* for 15 min., the precipitates were redissolved in water and samples removed for enzyme assay and for determination of protein N. The total supernatant activity and protein N were then calculated by difference. Direct enzyme assay of the supernatant activity in the presence of high concentrations of $(NH_4)_2SO_4$ was found to be unreliable.

pH *Measurement.* This was made with the glass electrode on the Cambridge pH meter.

Source of the enzyme

Rumen liquor was withdrawn from adult male castrated Cheviot sheep, with permanent rumen fistulae. Initially the animals' diet consisted of hay and concentrate (two parts linseed oil cake and one part crushed oats), with free access to water and salt lick. Later the concentrate was excluded. Much cleaner preparations were thereby obtained, without deterioration of the health of the animals or noticeable alteration of the glucuronide-decomposing activity of the whole rumen liquor. The daily diet then consisted of 750 g. hay fed at 7 a.m., and a further 750 g. hay fed at 4 p.m. The rumen liquor was withdrawn at 9.30 a.m., and for large-scale preparations the samples from several sheep were pooled, not more than 1500 ml. being withdrawn from a single sheep each time.

Preparation of cell-free extracts

The rumen liquor was first strained through six layers of surgical gauze to remove coarse food particles, and the suspension of micro-organisms was then fractionated by centrifuging to obtain the fraction which sedimented between the limits of 15 *g* for 2 min. and 1500 *g* for 30 min. This greenish sediment was washed with water on the centrifuge, and then contained 45–60 % of the apparent total enzyme activity of the strained rumen liquor. It was resuspended in 5 vol. water, and poured into 20 vol. of rapidly stirred acetone at 15°, and mechanical stirring was continued for a further 30 min. After being centrifuged at 1500 *g* for 6 min., the brown precipitate was washed on the centrifuge with a further 3 vol. acetone. (Omission of this washing yielded a dark brown mass which proved difficult to resuspend in water.) The product, after being dried *in vacuo* over $CaCl_2$, was a friable, almost white powder (yield 1 g./500–800 ml. strained rumen liquor).

The acetone-treated powder was suspended in water by homogenizing in a glass homogenizer (Umbreit, Burris & Stauffer, 1949) for 1 min. at 20°. The aqueous suspension was diluted to the original volume of the aqueous suspension of micro-organisms before acetone treatment, and stirred slowly at 20° for 4 hr.; it was then centrifuged at 8300 *g* for 30 min., and the supernatant recentrifuged under the same conditions. The final turbid supernatant was almost free of visible particles under the high-power light-microscope, and contained about 40 % of the total enzyme activity of the suspension of acetone-treated powder. Soluble-enzyme activity was defined as that which remained in this supernatant. In large-scale preparations a further quantity of soluble-enzyme activity (10–15 % of that already extracted) could be recovered by resuspending the cell debris in half the original quantity of fresh water and repeating the extraction procedure.

Preliminary extractions with 0·6N-Na_2HPO_4–0·3N citric acid mixed buffer (McIlvaine, 1921) over the pH range 4·3–8·0 indicated that whereas the ratio supernatant activity/suspension activity remained almost constant, there was appreciable loss of total enzyme activity in buffered solutions during the extraction period, particularly outside the pH range 6–7. Maximum supernatant activity resulted from extraction with water alone. Extractions with aqueous solutions of the non-ionic surface active agents Triton A 20 and Triton X 100 (Rohm & Haas Co., Philadelphia), at a concentration (0·003 %, w/v), which did not affect the subsequent enzyme assay, gave results similar to extraction with water. Maximum extraction with water was reached in approximately 2 hr. at 20°; subsequently the slow increase in enzyme extraction was almost nullified by the loss of total activity. Although the rate of enzyme inactivation in the aqueous suspension at 3° was less than at 20°, the extracted enzyme activity at the lower temperature was less, even after 72 hr. extraction. Attempts to render soluble a further part of the enzyme activity associated with the residual cell debris after aqueous extraction, by further treatment with acetone as described above, led to loss of more than 50 % of the residual activity of the debris.

Purification of the enzyme

Stage A. Precipitation of mucilaginous material. Precipitation of non-active mucilaginous material in the crude cell-free extract at pH 4·5, according to the procedure used by Karunairatnam & Levvy (1951), caused a slight increase in the specific activity (see Table 1), although little solid was precipitated. The product (enzyme *A*) contained 85–90 % of the enzyme activity of the crude extract.

Stage B. 1st fractionation with $(NH_4)_2SO_4$. An initial fractionation of enzyme *A*, between the limits of 20–80 % $(NH_4)_2SO_4$ saturation, precipitated all the enzyme activity (enzyme *B*). The solubility-test curves of the variation of enzyme *B* activity and protein N at varying $(NH_4)_2SO_4$ concentrations are

shown in Figs. 1*a* and 1*b* respectively. Precipitation of enzyme *B* activity and of protein did not commence below 30% $(NH_4)_2SO_4$ saturation, and were both completed at 75% saturation. Thus there was little non-overlapping non-active protein at this stage. The steeper gradient of Fig. 1*b* at commencement of precipitation, as compared with that of Fig. 1*a*, indicated the presence of less soluble non-active protein, with a similar effect above 65% $(NH_4)_2SO_4$ saturation due to more soluble non-active protein. These conclusions are more easily seen in the specific-property solubility test (Fig. 1*c*). In this curve the constant gradient between points *A* and *B*, at about 42 and 62% $(NH_4)_2SO_4$ saturation respectively, indicated the maximum purification which could be achieved by subsequent $(NH_4)_2SO_4$ fractionation of enzyme *B*. From Fig. 1*a* it can be seen that *ca.* 80% of the total enzyme *B* activity should be precipitated between these limits.

Stage C. 2nd fractionation with $(NH_4)_2SO_4$. The enzyme *B* preparation was fractionated between the limits of 42–60% $(NH_4)_2SO_4$ saturation, and after redissolving the precipitate in water and dialysing, solubility tests of the resulting enzyme (enzyme *C*) activity and protein N between the limits of 35–70% $(NH_4)_2SO_4$ saturation were performed. The specific-property solubility test for enzyme *C* is shown in Fig. 2. Much impurity is shown to be precipitated below 50% $(NH_4)_2SO_4$ saturation, with a linear range of maximum specific activity between the limits of 50–60% $(NH_4)_2SO_4$ saturation, which should yield a theoretical recovery of 60% enzyme activity.

Stage D. 3rd fractionation with $(NH_4)_2SO_4$. Enzyme *D* was obtained after fractionation of the enzyme *C* preparation between the limits 50–60% $(NH_4)_2SO_4$ saturation. The specific-property solubility test for enzyme *D* is shown in Fig. 3. It was found that neither enzyme activity nor protein N was precipitated below 45% $(NH_4)_2SO_4$ saturation. Overlapping impurity was still obvious at both ends of the precipitation range, and the curve shows a maximum specific activity gradient between the limits of 50–60% $(NH_4)_2SO_4$ saturation. Fractionation of enzyme *D* between these limits should result in 90% recovery of enzyme activity.

Stage E. 4th fractionation with $(NH_4)_2SO_4$. Since attempts to decrease the limits of fractionation to less than a difference of 10% $(NH_4)_2SO_4$ saturation were not very successful, the enzyme *D* preparation was again fractionated between the limits of 50–60% $(NH_4)_2SO_4$ saturation. In practice *ca.* 60% of the enzyme activity was recovered. The specific-property solubility test on the final enzyme solution (enzyme *E*) is shown in Fig. 4. This is an almost linear curve, extrapolating through the origin, indicating the apparent absence of more soluble

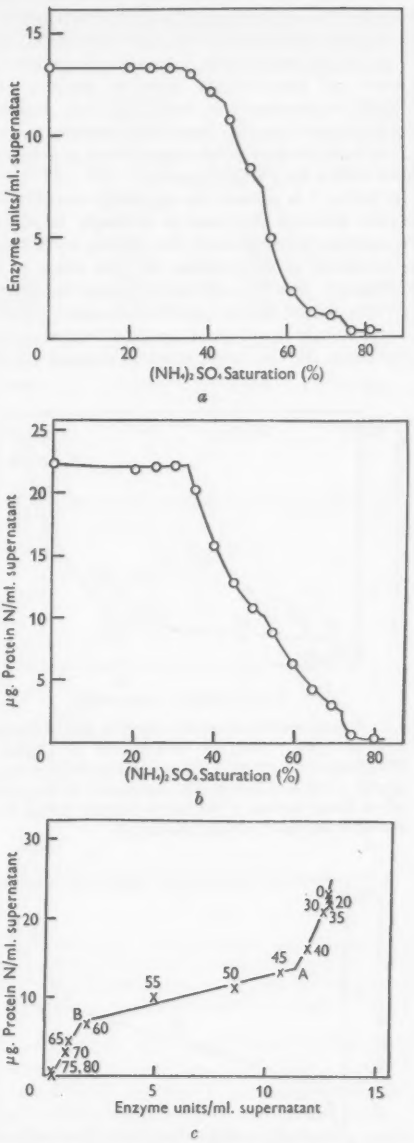

Fig. 1. *a*, *b*: precipitation of enzyme activity and protein N at varying concentrations of $(NH_4)_2SO_4$. Enzyme preparation *B* (Table 1). *c*: first specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *B*. $(NH_4)_2SO_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points *A* and *B* = 900 enzyme units/mg. protein N.

impurity. The experimental point corresponding to the original solution (i.e. 0% salt saturation) does not lie on the linear curve, indicating the continued presence of less soluble inactive protein. The difficulty experienced in removing this impurity suggests that it may be denatured enzyme.

The final product after stage *E* was a colourless liquid with a very slight opacity.

In Table 1 is shown the apparent recoveries of enzyme activity obtained in a single large-scale preparation, together with the specific activities of the solutions or suspensions at each stage of the purification. For the cell-free solutions the specific activities agreed closely with the estimates given by the previously conducted specific-property solubility tests. In the preparation illustrated the loss of enzyme activity during the initial centrifugal fractionation of the rumen liquor was higher than was normally found. The recovery of enzyme activity in the initial cell-free extract was 17·5% of that in the strained rumen liquor, with a ninefold enzyme purification (calculated in total N content). The recovery of enzyme *E* activity was 10·5% of that in the initial crude cell-free extract, with a 42-fold purification (calculated on protein N). The apparent overall enzyme purification from the strained rumen liquor to enzyme *E* was thus 378-fold.

Other preparations, in which $(NH_4)_2SO_4$ fractionations were carried out between the same limits as in Table 1, but not as far as stage *E*, gave values of the specific activities at the various stages similar to those given in Table 1.

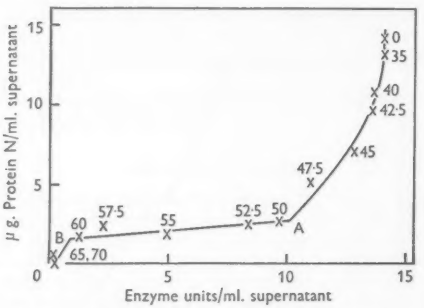

Fig. 2. Second specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *C* (Table 1). $(NH_4)_2SO_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points *A* and *B* = 9000 enzyme units/mg. protein N.

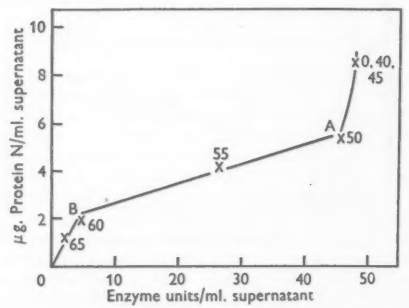

Fig. 3. Third specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *D* (Table 1). $(NH_4)_2SO_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between points *A* and *B* = 12000 enzyme units/mg. protein N.

Test for glucuronide-decomposing enzymes in the diet

Appreciable amounts of pigmented food particles present in normal rumen liquor are sedimented during the fractional centrifuging of the liquor, and these cannot readily be separated from the micro-organisms present in the various fractions (Davidson, 1954). There was, however, no liberation of phenolphthalein when phenolphthalein glucuronide (0·00125M) was incubated at 38° and pH 6·1 with suspensions of ground hay of variable particle size up to *ca.* 0·5 mm. diameter, before or after acetone treatment as described above. It was concluded that none of the glucuronide-decomposing enzyme activity occurring in the rumen could have originated in the feed.

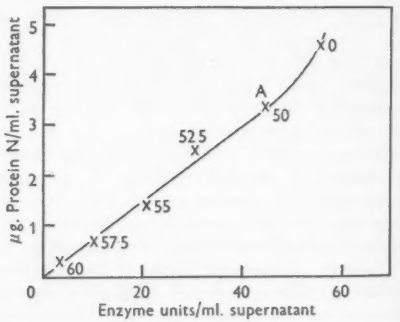

Fig. 4. Fourth specific-property solubility test (Falconer & Taylor, 1946). Enzyme preparation *E* (Table 1). $(NH_4)_2SO_4$ concentration (% saturation) for each experimental point shown on graph. Reciprocal of the gradient of the linear section of the curve between the origin and point *A* = 13600 enzyme units/mg. protein N.

Table 1. *Stages in the purification of the rumen glucuronide-decomposing enzyme*

Substrate 0·0005 M phenolphthalein β-D-glucuronide. Unit of enzyme activity as specified in Methods section.

Stage	Vol. (ml.)	Enzyme activity		Nitrogen content (total or protein N) (μg. N/ml.)	Specific activity (units/mg. N)	Theoretical specific activity from Figs. 1*c*, 2, 3 (units/mg. N)
		Units/ml.	Total units			
Strained rumen liquor	7 600	33·4	257 000	1440*	23	—
Active fraction of micro-organisms	424	220	93 400	2530*	87	—
Cell-free extract of acetone-dried powder	1 120	40	44 800	202* 139†	198 288	—
Enzyme *A* (after pptn. at pH 4·5)	1 140	36	41 200	113†	318	—
Enzyme *B* (after 1st $(NH_4)_2SO_4$ fractionation)	635	42	26 700	128†	325	—
Enzyme *C* (after 2nd $(NH_4)_2SO_4$ fractionation)	101	163	16 500	173†	950	900
Enzyme *D* (after 3rd $(NH_4)_2SO_4$ fractionation)	60	150	9 000	18·2†	8 300	9 000
Enzyme *E* (after 4th $(NH_4)_2SO_4$ fractionation)	11·5	408	4 700	33·8†	12 000	12 000

* Total N. † Protein N.

Effects of varying the buffer, enzyme concentration and period of assay

Free inorganic phosphate was rigorously excluded from an enzyme *C* preparation after extraction of the acetone-treated powder; 0·1 N citric acid–NaOH buffer, pH 6·1, was employed before each $(NH_4)_2SO_4$ fractionation. After the final dialysis, determination by the procedure of Fiske & Subbarow (1925) gave a free phosphate content of less than 0·2 μg. P/ml. Assays of enzyme activity of this preparation at pH 6·1 in 0·3 N-H_3PO_4–NaOH, 0·1 N citric acid–NaOH and 0·2 N phthalic acid–NaOH buffers gave identical values.

The amount of phenolphthalein liberated in 1 hr. at 38° and pH 6·1 from 0·0005 M phenolphthalein glucuronide, using the usual enzyme preparation in phosphate buffer, was directly proportional to the concentration of enzyme *C*. The reaction velocity, with constant enzyme concentration, remained unchanged after 4 hr. incubation at 38° in phosphate buffer, pH 6·1.

These results confirm the findings of Karunairatnam & Levvy (1951); their significance is discussed later.

Variation of enzyme activity with pH

The variation of enzyme activity with pH, using 0·00125 M phenolphthalein glucuronide as substrate, in 0·6 N-Na_2HPO_4–0·3 N citric acid mixed buffer, pH 4·5–8·5, was studied using the procedure of Karunairatnam & Levvy (1951). It was found (Fig. 5) that the enzyme showed only a single optimum, but this varied slightly with the purity of the enzyme preparation. After the first $(NH_4)_2SO_4$ fractionation (enzyme *B*), optimum enzyme activity was at pH 6·0–6·1, as found by Karunairatnam & Levvy (1951), whereas after a further $(NH_4)_2SO_4$ fractionation (enzyme *C*) the optimum shifted to pH 6·5–6·6.

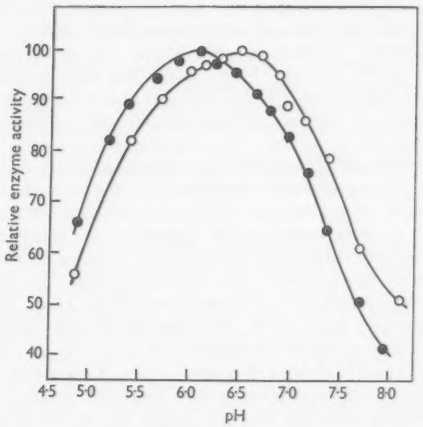

Fig. 5. Variation of rumen enzyme activity with pH. Substrate 0·00125 M phenolphthalein glucuronide; assays in 0·3 N-H_3PO_4–NaOH buffer. ●—●, enzyme preparation *B*; ○—○, enzyme preparation *C* (Table 1).

The liberation of phenol from 0·015 M-phenyl glucuronide in 0·05 N citric acid–NaOH buffer in 1 hr. at 38° by enzyme *C* preparations showed a single optimum at pH 6·6, i.e. almost identical with that obtained with phenolphthalein glucuronide as substrate and enzyme *C*.

Variation of enzyme activity with substrate concentration

Fig. 6*a* shows the variation of enzyme activity with the concentrations of the substrates phenolphthalein and phenyl β-D-glucuronides in typical experiments. For phenolphthalein glucuronide,

inhibition by excess substrate was evident at concentrations above 0·005 M, whereas there was no evidence of the attainment of maximum enzyme activity even at the highest concentration (0·02 M) of phenyl glucuronide used. Enzyme *C* preparations were employed in each case.

Analysis of these results by the graphical methods of Lineweaver & Burk (1934) and of Hofstee (1952) showed some anomalies in the kinetics. The plots of $1/v$ against $1/S$, S/v against S and v against v/S (Fig. 6*b*), where v represents the initial reaction velocity (enzyme activity) and S the substrate concentration, gave non-linear curves at all substrate levels in each case, whereas according to Michaelis–Menten theory these curves should be linear in the absence of substrate inhibition. The effect obtained was one of enzyme activation at increasing substrate concentration in the lower range; thus in Fig. 6*b* there is an increase in the gradient of the curve as the substrate concentration increases (i.e., v/S decreases) with a sudden reversal of gradient in the case of phenolphthalein glucuronide due to the subsequent effect of substrate inhibition. Statistical advice (from Mr A. W. Boyne of this Institute) was that no true straight line could be fitted to the experimental points at concentrations below the inhibition level.

This made impossible the determination of K_s or K_2, the respective dissociation constants of the active binary enzyme–substrate complex ES and the inactive complex ES^n formed in the region of substrate inhibition, or of n, the number of substrate molecules in the latter complex, by the graphical method of Lineweaver & Burk (1934). The apparent value of K_s for phenolphthalein glucuronide was therefore determined from Fig. 6*a*, for from the Michaelis–Menten theory, K_s is equal to the substrate concentration required to give a reaction velocity of $V_m/2$, where V_m is the theoretical maximum reaction velocity. Values of K_s for phenolphthalein glucuronide obtained in different experiments are shown in Table 2, the mean values being $7{\cdot}7 \times 10^{-5}$ M at pH 6·5, and $6{\cdot}0 \times 10^{-5}$ M at pH 6·1.

Since the maximum reaction velocity for the substrate phenyl glucuronide was not reached, the value of K_s for this compound was measured by the indirect method described below.

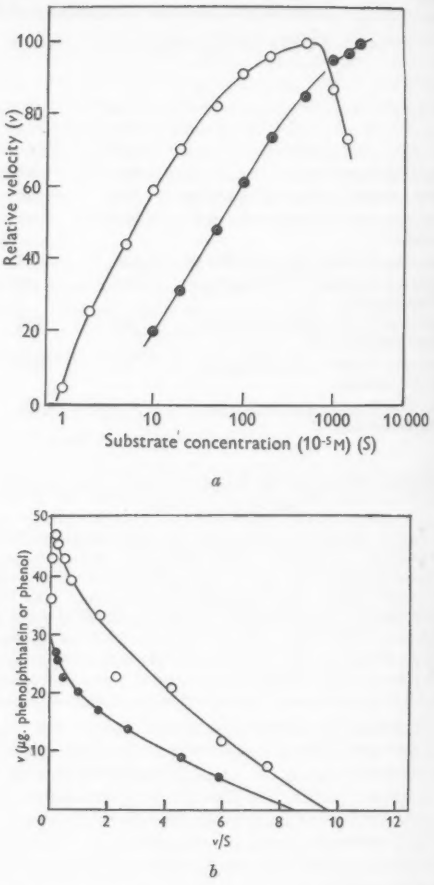

Fig. 6. *a*: effect of varying the substrate concentration (S) on the rate of decomposition (v) of phenolphthalein and phenyl glucuronides by enzyme preparation *C* (Table 1). ○—○, Decomposition of phenolphthalein glucuronide in 0·3 N-H_3PO_4–NaOH buffer, pH 6·5; ●—●, decomposition of phenyl glucuronide in 0·05 N citric acid–NaOH, pH 6·6. *b*: results plotted according to Hofstee (1952). For decomposition of phenolphthalein glucuronide (○—○), v/S in units of 10^5 μg./hr. For decomposition of phenyl glucuronide (●—●), v/S in units of 10^4 μg./hr.

Table 2. *Apparent Michaelis constants of substrates for the rumen enzyme*

Substrate	Constant	Values obtained M 10^{-5}	pH	Apparent relative affinity (mean)
Phenolphthalein β-D-glucuronide	K_s	6·3, 9·1 5·0, 3·7, 9·5, 6·0	6·5 6·1	1
Phenyl β-D-glucuronide	$K_s(=K_s)$	220, 320	6·1	0·022
(−)-Menthyl β-D-glucuronide	$K_s(=K_s)$	260, 320	6·1	0·021

Table 3. *Recovery of glucuronic acid after addition to enzyme C preparations, with and without incubation at 38° for 5 hr. in 0·05 N citric acid–NaOH buffer, pH 6·5. Protein pptn. with 5 % trichloroacetic acid*

Glucuronic acid added (μg.)	Glucuronic acid recovered (μg.)			
	Non-incubated		After incubation	
	− Enzyme	+ Enzyme	− Enzyme	+ Enzyme
250	238	182	253	185
300	306	249	306	239

Measurements of reducing sugar

Attempts were made to measure the liberation of reducing sugar from the enzymic decomposition of 0·01 M (−)-menthyl β-D-glucuronide, using concentrated enzyme preparations (enzyme *C*, after 48 hr. dialysis). Although there was evidence of the liberation of reducing sugar at pH 5·5–7·0, the amounts measured, estimated as glucuronic acid and using periods of incubation at 38° of up to 4 hr., were invariably very low. Measurement of glucuronic acid recovery after addition of this compound to enzyme preparations showed, however, an apparent loss (*ca.* 30 %) as shown in Table 3. There was no further change in the recovery of glucuronic acid after incubation at 38° for 5 hr., suggesting that the apparent loss was due to adsorption by and co-precipitation with protein or polysaccharide, and not to enzymic destruction or to the liberation from the substrate of a free sugar with a reducing power widely different from that of glucuronic acid.

It was concluded that (−)-menthyl β-D-glucuronide was a substrate for the rumen enzyme, but the kinetics of enzyme action on this substrate could not be measured directly. The indirect determination of K_s is described below.

Inhibition of phenolphthalein glucuronide decomposition by alternative substrates

The effects of 0·01 M phenyl and (−)-menthyl glucuronides on the enzymic decomposition of phenolphthalein glucuronide were studied. In such experiments these alternative substrates acted as competitive inhibitors of the enzyme, and it was assumed that their concentrations remained virtually unchanged throughout the incubation period. Assays were conducted in phosphate buffer at pH 6·1, using enzyme *C* preparations; Fig. 7 shows the variation of enzyme activity (liberation of free phenolphthalein) with phenolphthalein glucuronide concentration, in the absence and presence of each alternative substrate.

By considering these alternative substrates as competitive inhibitors, their affinities for the enzyme can be determined from the Michaelis equation for a competitive system.

$$v = \frac{V_m S}{K_s (1 + I/K_i) + S},$$

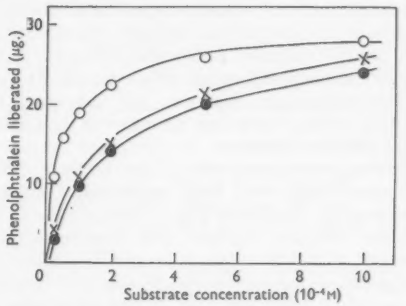

Fig. 7. Effect of standard substrate concentration on the liberation of phenolphthalein from phenolphthalein glucuronide in absence and presence of (*a*) 0·01 M phenyl β-D-glucuronide, (*b*) 0·01 M (−)-menthyl β-D-glucuronide, in 0·3 N phosphate buffer, pH 6·1. O—O, No alternative substrate present; ●—●, phenyl β-D-glucuronide present; ×—×, (−)-menthyl β-D-glucuronide present.

where v is the initial reaction velocity (enzyme activity) with substrate concentration S and inhibitor concentration I, and V_m the theoretical maximum reaction velocity (see Levvy & Marsh, 1954). The apparent values of K_s and $(1 + I/K_i)$ were obtained directly from Fig. 7, being equal to the substrate (phenolphthalein glucuronide) concentration required for half maximum reaction velocity of the respective non-inhibited and inhibited reactions, V_m being unchanged. The values of K_i, the Michaelis constant of the competitive inhibitor, for phenyl and (−)-menthyl glucuronides, were then calculated, and the values obtained from different experiments are shown in Table 2. The value of K_i should be identical with that of K_s for the second substrate, for both in this case represent the dissociation constant of the enzyme–alternative substrate complex. Lederberg (1950) has used this method of indirect determination of K_s of various substrates for the β-galactosidase of *Escherichia coli*.

It is seen from Table 2 that the values of K_i (or K_s) for phenyl and (−)-menthyl glucuronides were approximately equal (*ca.* 3×10^{-3} M). The affinity of the rumen enzyme for phenolphthalein glucuronide is thus considerably higher than for the other substrates examined.

DISCUSSION

The degree of purification achieved with the rumen enzyme compares unfavourably with that of animal tissue β-glucuronidase obtained recently. Thus using different techniques, Sarkar & Sumner (1950), Bernfeld & Fishman (1950) and Smith & Mills (1953) have each obtained an approximately 1000-fold purification of the tissue enzyme, whereas after preparation of the cell-free extracts, a 40-fold purification of the rumen enzyme was obtained, mostly in the ammonium sulphate fractionations. The method adopted would seem valuable in the earlier stages of purification before the use of a more specific technique, however, by indicating the limiting purity which may be achieved by a multi-stage salt fractionation.

In the preparation given in Table 1, the protein N recovered after the final ammonium sulphate fractionation was about 2% of that present in the original cell-free extract. The specific activity gradient of 13600 units/mg. protein N obtained for the purified rumen enzyme in the final specific-property solubility test may be compared with the values for highly purified tissue β-glucuronidase, e.g. 200000 units/mg. protein N for ox-liver β-glucuronidase (Smith & Mills, 1953); the rumen enzyme has a higher affinity for phenolphthalein glucuronide than has animal β-glucuronidase. It seems unlikely that the final product represents the rumen enzyme in a high state of purity. Falconer & Taylor (1946) have pointed out that in circumstances where protein impurities may have identical solubilities with those of the enzyme studied, a linear specific-property solubility test is not a criterion of absolute purity; it also appears possible that a homogenous solid solution of enzyme and inactive protein is precipitated.

Since the rumen enzyme is wholly intracellular, assay of the enzyme activity of a suspension of the intact micro-organisms cannot be compared quantitatively with that of a suspension of the acetone-treated powder or with subsequent stages in which the enzyme is fully soluble, and the recoveries given for the earlier stages of purification are probably inaccurate. Strains of haemolytic streptococcus containing a glucuronide-decomposing enzyme have shown a 5- to 10-fold increase in activity after sonic disintegration (Jacox, 1953), and with the rumen enzyme a slightly higher total activity was noted after acetone treatment. Assays of enzyme activity in the original rumen liquor are even less reliable, since decomposition of the standard substrate may well be inhibited competitively by natural substrates present in the liquor.

Endogenous inhibitors appear to be absent from partially purified cell-free preparations, however, since there was found a linear variation of enzyme activity with enzyme content (see Levvy & Marsh, 1954); Karunairatnam & Levvy (1951) found no evidence of a thermostable coenzyme in cell-free extracts of the rumen enzyme, and their results may also be interpreted as indicating the absence of a thermostable inhibitor, e.g. one similar to that found in rat-liver β-glucuronidase preparations (Walker & Levvy, 1953).

There is a fraction of the enzyme activity closely associated with the insoluble disrupted cell contents which, although fully active, cannot easily be brought into solution. Thus aqueous extraction after acetone treatment removed only 45% of the total enzyme activity; Karunairatnam & Levvy (1951) obtained an extraction of 57% of the activity from cells disintegrated by mechanical treatment.

The single pH optimum obtained with different preparations of the rumen enzyme at 6·1 or 6·5 may be compared with values given for similar enzymes of microbial origin, e.g. those from *Escherichia coli* (Buehler, Katzman & Doisy, 1949; Smith & Mills, 1950), pH-optimum 6·2, and from *Streptococcus haemolyticus* (Jacox, 1953), pH-optimum 5·5–7·0. Animal tissue β-glucuronidases have a pH optimum at 4·5 and some an additional optimum at pH 5·0–5·2; baicalinase, the only plant β-glucuronidase which has been investigated, has a single pH optimum varying between pH 3·8 and pH 4·5, dependent upon the buffer (Levvy, 1954). This supports the experimental evidence that the rumen enzyme is of microbial origin and does not originate from plant particles in the diet or secretion of tissue β-glucuronidase into the rumen by the host animal.

The phenolphthalein glucuronide concentration (0·005M at pH 6·5) for maximum enzyme activity was higher than the value of 0·00062M at pH 6·1 obtained by Karunairatnam & Levvy (1951). This, and also the slight shift in the pH optimum in enzyme preparations of different stages of purity, may be due to the removal of interfering substances by ammonium sulphate fractionation; Walker & Levvy (1953) observed that in the absence of its endogenous inhibitor the optimum substrate concentration for rat-liver β-glucuronidase preparations was almost doubled. The difficulties found here in applying the graphical methods of Lineweaver & Burk (1934) to the experimental results were not experienced by Karunairatnam & Levvy (1951), or generally by other workers with animal tissue β-glucuronidase, but Fishman (1939*b*) has obtained anomalous curves of enzyme activity and substrate concentration for purified ox-spleen β-glucuronidase somewhat similar to those obtained by the author. A possible explanation of the activating effect of the enzyme at moderate substrate concentrations may be that, apart from an active binary complex ES, other active complexes $ES^{n'}$ may also be formed. Hofstee (1952) has shown that the same effect may

be caused by the simultaneous action of two similar enzymes of differing affinities for the same substrate. In view of the complications of both apparent activation and inhibition by excess substrate, the apparent values of K_s and of K_i ($=K_s$) for the alternative substrates, obtained by direct graphical methods, represent the most accurate values obtainable from the data.

The affinity ($1/K_s$) of phenolphthalein glucuronide for the rumen enzyme was found to be somewhat higher than for animal β-glucuronidase (e.g. see Levvy, 1952); the much lower affinities of phenyl and (−)-menthyl glucuronides were approximately equal, and of the same order, viz. $1/K_s = 300\,\text{M}^{-1}$ for both enzymes. The rumen enzyme differs from mouse-liver β-glucuronidase (Levvy & Marsh, 1952) in that there was no apparent activation of the decomposition of phenolphthalein glucuronide by (−)-menthyl β-D-glucuronide.

The mode of action of the rumen enzyme, e.g. whether or not it is a true β-glucuronidase, has still to be determined. The fact that an enzyme preparation free from inorganic phosphate gave identical assays of enzyme activity in different buffers makes it unlikely, however, that the enzymic liberation of phenolphthalein from its glycosiduronic acid takes place by a mechanism of phosphorolysis. An investigation of the reaction products of enzyme action, and of the specificity and inhibition of the rumen enzyme, will be published later.

SUMMARY

1. A partial purification of the glucuronide-decomposing enzyme associated with the medium-sized micro-organisms in the sheep rumen is described. This was achieved by aqueous extraction of an acetone-treated powder and acid precipitation of inactive material, followed by repeated fractional precipitation with ammonium sulphate. The fractionation limits were controlled by use of the specific-property solubility test of Falconer & Taylor (1946).

2. The overall purification (in terms of enzyme activity/mg. N) which was achieved, commencing with strained rumen liquor, was approximately 400-fold.

3. The single pH optimum of enzyme activity varied slightly with the state of purity of the preparation between the limits 6·1 to 6·6 and was the same for phenyl and phenolphthalein glucuronides.

4. The effect of varying substrate concentration was studied and certain anomalies are discussed.

The author wishes to express his gratitude to Dr G. A. Levvy for his constant interest and helpful criticism in this work, and to thank Dr Dorothy Duncan for the insertion of permanent rumen fistulae, and Mr G. Pratt for technical assistance.

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The Immobilization of Spermatozoa by Freezing and Thawing and the Protective Action of Glycerol

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(*Received* 3 *June* 1954)

It was discovered by Polge, Smith & Parkes (1949) that spermatozoa could be frozen and thawed without loss of motility if glycerol was included in their suspending medium. Following this discovery the spermatozoa of the domestic fowl (Polge, 1951), of the bull (Polge & Rowson, 1952) and of the herring (Blaxter, 1953) have been preserved without loss of function for long periods in the frozen state. The importance of the practical applications of this method has been discussed (Polge & Parkes, 1952). The investigation of the protective action of glycerol was facilitated by the observation that red blood cells also were protected against the adverse effects of freezing and thawing by means of glycerol (Smith, 1950), and the nature of the damage occurring to red blood cells when they are frozen and thawed, and of the protection given by glycerol was then described (Lovelock, 1953*a*, *b*). Briefly, haemolysis by freezing and thawing is due to the destructive action of the concentrated salt solution to which the cells are exposed when water is removed as ice. In the presence of glycerol the electrolyte concentration at temperatures below the freezing point is sufficiently reduced to explain in full the protection afforded by glycerol.

The apparent simplicity of the processes causing the haemolysis of red blood cells by freezing and thawing, and of the protective action of glycerol, made it of some interest to observe the behaviour of other living cells on freezing and thawing. It seemed that this provisional explanation of the effects of freezing could best be tested using living cells with a wide range of sensitivity to hypertonic salt solutions. It also seemed desirable to use a criterion of damage more delicate than haemolysis.

Both of these requirements are met by the spermatozoa of different animals. Those of the rabbit are particularly sensitive to hypertonic salt solutions (Emmens, 1948), while those of the herring clearly will tolerate salt solutions at least as strong as sea water. In addition, the observation of the motility of spermatozoa, even if somewhat lacking in precision, provides a different and sensitive criterion for the assessment of freezing damage.

This paper records the effects of freezing and thawing upon the motility of the spermatozoa of the rabbit, the bull, the fowl and the herring. The modification of these effects by glycerol is also described.

METHODS

Semen. Rabbit and bull semen were collected using the techniques described by Macirone & Walton (1938) and Walton (1942). After collection, the semen contained in a glass test tube was placed in a 250 ml. glass beaker filled with water at 25°. The beaker was then placed in a refrigerator and left for 2 hr. to cool to 2°. This slow-cooling procedure was necessary because the spermatozoa of both the bull and the rabbit are sensitive to thermal shock. The semen was kept at 2° or in crushed ice at 0° until required for use, but was always used within 5 hr. of collection.

Fowl semen was collected by the method described by Burrows & Quinn (1935). After collection the semen was placed directly in a cold-room at 2°, and kept there until ready for use. Herring sperm were expressed directly from the gonads. These had been dissected from the herring and stored at 0° for not more than 2 days.

Suspending media. Before each experiment the semen was diluted with 9 vol. of the appropriate suspending medium at 0°. This was partly because the assessment of motility was easier with diluted semen, and partly to define the principal electrolytes of the cell suspension. The suspending media, which were kept at 0°, were:—rabbit: NaCl, 0·12 M; Na_2HPO_4, 0·01 M; KH_2PO_4, 0·01 M; glucose, 0·025 M; egg yolk 10%;—bull: trisodium citrate, 0·09 M; KH_2PO_4, 0·01 M; egg yolk 10%;—fowl: NaCl, 0·15 M; Na_2HPO_4, 0·01 M; KH_2PO_4, 0·01 M;—herring: sea water. When glycerol was used, it was included in the medium before mixing. Semen was mixed directly with media containing 0·5 M glycerol or less, but with higher glycerol concentrations the cells were left for 30 min. in a medium containing 0·5 M glycerol at 0°, and then diluted with medium containing sufficient glycerol to provide the desired final concentration. After every addition of glycerol the cells were left for 30 min. to ensure their permeation by the solute.

Measurement and maintenance of low temperatures. In all experiments temperatures were measured in terms of the e.m.f. generated by a calibrated copper–constantan thermocouple. Temperatures between 0 and −30° were obtained by means of a thermostatically controlled ethanol bath which was maintained at the required temperature within ±0·1°. Temperatures below −30° were obtained by means of a bath of ethanol (10 l.) contained in an open-necked Dewar flask and adjusted to the required temperature by the addition of solid CO_2. Under the conditions used the temperature remained constant during the 10 min. required for the experiments.

Assessment of motility. The cells were examined microscopically as a thin preparation between slide and cover glass, with a 16 mm. objective. The microscope was enclosed within a chamber kept at 37°, in which the preparation was left for 2 min. before examination. The motility of the spermatozoa under test was compared with the motility of the untreated controls kept at 0° and was expressed as an arbitrary score. Motility equal to that of the control was given a score of 10 and complete immobility a score of 0.

Procedure for exposing spermatozoa to concentrated salt solutions. Semen (0·1 ml.) was added to concentrated salt solution (0·3 ml.) contained in a small test tube at 0°. After 10 min. the mixture was diluted with 0·6 ml. of a mixture of the normal suspending medium and distilled water of such composition as would give a final concentration equal to that of the normal suspending medium. The diluted cell suspension was then warmed and examined for motility. The salt concentration above which a progressive fall in motility took place was recorded as that causing irreversible damage to the spermatozoa in 10 min. at 0°.

Procedure for freezing suspensions of spermatozoa. All experiments were made in flat-bottomed tubes (100 × 5 mm., wall thickness 1 mm.). The cell suspension (0·2 ml.) in one of these tubes was placed in a bath set at the required temperature. The usual procedure was to leave the suspension in the cold bath for 10 min. after freezing had taken place either spontaneously or by seeding with an ice crystal. With spermatozoa of the bull and of the rabbit a slightly different procedure was used in experiments below −10° to try to avoid thermal shock. The cells were first cooled to −10° and left there unfrozen for 10 min. (Supercooling of small volumes to this temperature is not difficult.) The unfrozen suspension was then transferred to a bath at the required temperature and there frozen and left for a further 10 min. After all experiments the cells were thawed by immersion in a water bath at 20°.

The choice of 10 min. for exposure to freezing was based upon pilot experiments and experience with other cells. These showed that destruction occurs rapidly during the first 3 min. after freezing but then slows considerably. In 10 min. most of the damage due to freezing has taken place.

RESULTS

Fig. 1 shows the depression of motility of the spermatozoa after freezing for 10 min. at temperatures between 0 and −45°. The effect of including various concentrations of glycerol in the suspending medium before freezing is also shown.

In Table 1 are the concentrations of the electrolytes used for suspending the cells, and the maximum concentrations which the cells will withstand at 10 min. at 0° without suffering some irreversible damage. Also shown are the freezing point of the latter solution, and the temperature below which the cells cannot be taken in the frozen state without suffering permanent damage. Corresponding values for human red blood cells are given for comparison.

DISCUSSION

Between 0 and −50° there is a critical region of temperature in which living cells perish if left for more than a few seconds. The precise bounds of this region for the human red blood cell are from −3 to −40° (Lovelock, 1953*a*). The protective action of glycerol is shown principally by its ability to reduce the extent of this critical region and, if sufficient is used, to remove it altogether. The results show that there are similar critical ranges of temperature in which spermatozoa suffer damage and that glycerol reduces the extent of these critical regions. The bounds of the critical regions and the temperatures at which the greatest damage takes place differ with the cells of different species.

The experimental results summarized in Table 1 show that the spermatozoa of the rabbit, bull and fowl are all more sensitive to freezing than the human red blood cell. On the other hand, herring spermatozoa are remarkably resistant to freezing; even in the absence of glycerol they are not damaged by freezing until the temperature falls below −6°, and are not destroyed completely until −15°.

The spermatozoa of the bull and of the rabbit are well known to be sensitive to thermal shock. This effect is described in detail in a recent review (Smith, 1954). The results (Fig. 1) show that for bull and rabbit spermatozoa no damage attributable to thermal shock took place down to −12°. Below this temperature the risk of damage by thermal shock increases as the temperature interval through which the cells are cooled becomes greater. At the lowest temperatures investigated, −20 to −30°, there is some chance that the observed death of cells may have been due to thermal shock as well as to the adverse effects of freezing and thawing. In the practical preservation of bull spermatozoa at −78° it is possible by using an adequate concentration of glycerol to cool sufficiently slowly to avoid damage by thermal shock. In the present experiments, however, which included freezing with low concentrations of glycerol, cooling at the rate found satisfactory for practical preservation would have been too slow. This is because the investigation of the lower limits of the critical region requires the passage of the cells through the rapidly destructive intermediate temperatures. Slow cooling through this unfavourable region would have completely obscured the effects of exposure to the lower temperatures.

The damage suffered by human red blood cells during freezing and thawing is due almost exclusively to the concentration of the electrolytes of the cell and their suspending medium as water is removed as ice. The results shown in Table 1 indicate that with all the spermatozoa tested the temperature at which damage first takes place is within 0·5° of the freezing point of a salt solution whose concentration is just sufficient to cause some irreversible damage. The concentration of electrolyte above which irreversible damage to the cell takes place appears to be characteristic for each

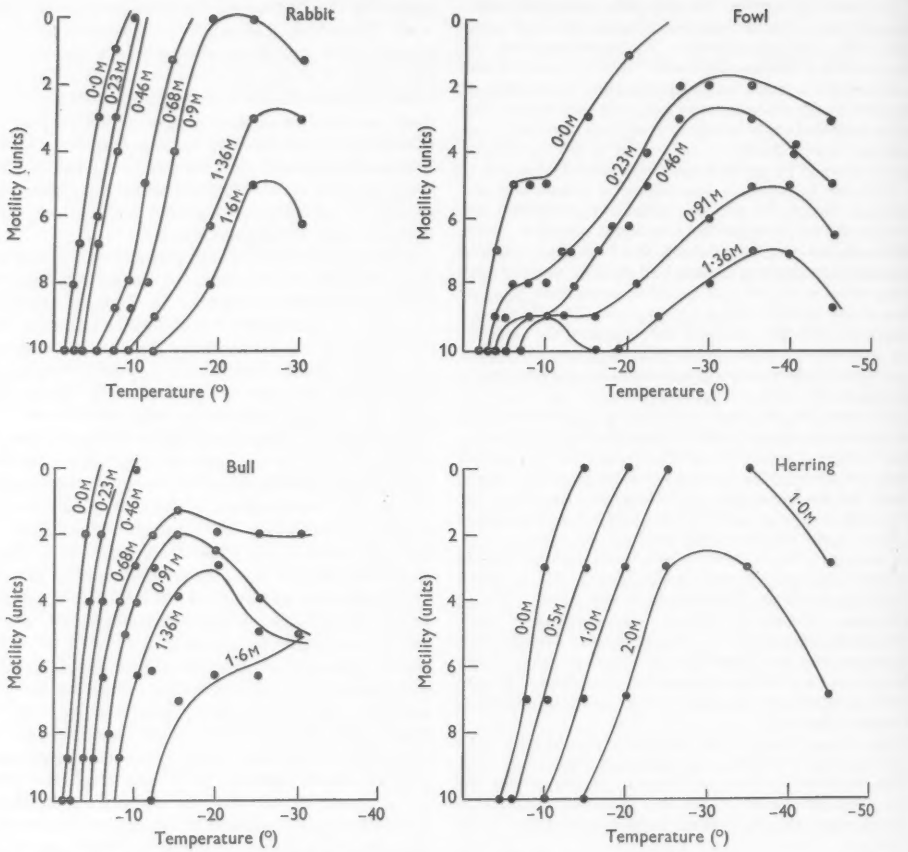

Fig. 1. The immobilization of spermatozoa of various species when frozen for 10 min. at the temperatures indicated. The cells were frozen in the media described in Table 1, and also in these media together with the concentrations of glycerol shown on each curve. The ordinates indicate the motility of the spermatozoa in the arbitrary units described in the text.

Table 1. *A comparison of the observed and calculated values for the temperature below which spermatozoa suffer irreversible damage when frozen*

Measurements were made of the temperature, ΔT_D, below which the cells suffered some irreversible damage, when frozen for 10 min. Measurements were also made of the electrolyte concentration, x_D, above which irreversible damage occurred when the cells were kept in it for 10 min. at 0° and then transferred back to their normal suspending medium. The calculated value of ΔT_D is the freezing point of a solution whose concentration is x_D.

Cell species	Suspending medium	Electrolyte concentration, x, of medium (M)	x_D (M)	ΔT_D Calculated (°)	ΔT_D Observed (°)
Rabbit spermatozoa	NaCl	0·12	0·32	−1·2	−1·0
Bull spermatozoa	Sodium citrate	0·09	0·18	−1·5	−1·5
Fowl spermatozoa	NaCl	0·15	0·35	−1·5	−2·0
Herring spermatozoa	Sea water	0·68	1·7	−6·2	−6·0
Human red blood cells	NaCl	0·15	0·9	−3·5	−3·0

type of cell. The herring spermatozoa which are normally exposed to sea water are very much more tolerant to increases in salinity than are the mammalian cells.

It follows from the colligative properties of solutions that the addition of a neutral solute such as glycerol will lower the concentration of salt in equilibrium with ice at any temperature below freezing. With the human red blood cell it has been shown that the protective action of glycerol is entirely due to this effect, and a simple relationship indicating the concentration of glycerol required to prevent damage to cells when they are frozen was deduced (Lovelock, 1954). This relationship can now be expressed

$$y = \frac{\Delta T_D - 2Kx_D}{Kx_D/x} = \left(\frac{\Delta T_D}{Kx_D} - 2\right) x,$$

where y = the concentration of glycerol required to prevent irreversible damage, x = the concentration of electrolyte in the suspending medium, nx (Lovelock, 1954) $= x_D =$ the electrolyte concentration at which damage first occurs, ΔT_D = the depression of the temperature below 0° at which damage first takes place, and K = a constant, indicating the observed depression of the freezing point of an aqueous solution by unit solute concentration.

It is clear that, since x_D is characteristic for each species of cell, the glycerol concentration required to protect a given species against damage by freezing will vary directly with the salt concentration of the medium used to suspend the cells before freezing.

Fig. 2 shows lines drawn according to this relationship using the values of x and x_D in Table 1 for each of the kinds of spermatozoa tested. The lines indicate the least concentrations of glycerol required by theory to protect the cells against irreversible damage on freezing to various temperatures. The points correspond to the observed glycerol concentrations found necessary in practice to protect the cells. Considering the inevitable lack of precision in the visual estimation of the motility of spermatozoa, the agreement between theory and practice is good for all of the spermatozoa tested. The experimental evidence offers strong support for the suggestion that the first cause of the death of spermatozoa on freezing is the increase of the concentration of their suspending medium to destructive levels. The protective action of glycerol can be explained completely in terms of its ability to prevent this increase.

On this basis it is clear that a relatively high concentration of glycerol is needed to protect herring spermatozoa, in spite of their great resistance to damage by freezing. With these cells their suspending medium, sea water, is already highly concentrated, and this presents a handicap to their successful preservation using glycerol. Herring spermatozoa will tolerate diluted sea water, and if they are frozen in this medium very much less glycerol is required than in sea water.

Red blood cells are protected by glycerol during freezing and thawing only when they are fully permeated by it. There is no benefit in preventing the electrolyte concentration of the suspending medium from rising if that of the cell interior is allowed to increase unchecked. No direct observations of the permeation of spermatozoa by glycerol

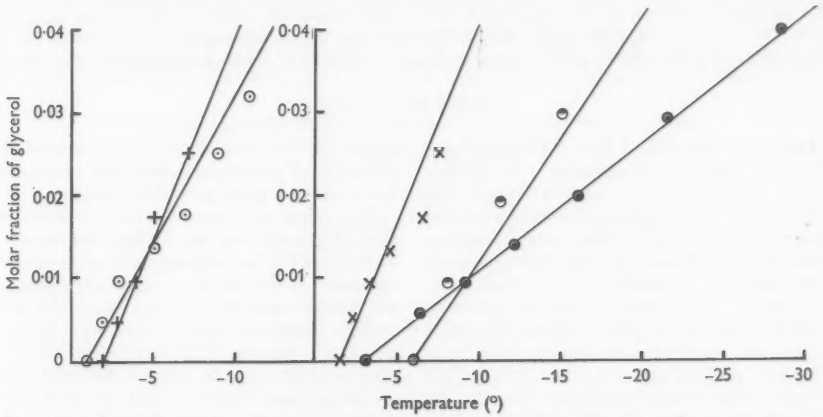

Fig. 2. The relationship between glycerol concentration expressed as a molar fraction and the temperature to which spermatozoa can be frozen without loss of motility. The solid lines indicate the glycerol concentrations required by theory, and the points the concentrations found necessary in practice. Spermatozoa: ⊙, rabbit; ×, bull; +, fowl; and ◓, herring. The theoretical line and experimental points for the human red blood cell, ●, using haemolysis as a criterion of damage, are also shown.

were made. All species of spermatozoa tested, including those highly sensitive to an increase in osmotic pressure, were fully motile in 2·0 M glycerol. It is most unlikely that cells impermeable to glycerol would retain motility in the presence of such an excessive external osmotic pressure, and the retention of motility in the presence of high concentrations of glycerol was taken as evidence that the cells were permeated by this solute. Other neutral solutes can to some extent protect spermatozoa against damage by freezing and thawing (Smith & Polge, 1950), but none is as effective as glycerol.

SUMMARY

1. The spermatozoa of the rabbit, the bull, the fowl and the herring were subjected to freezing and thawing in media containing various concentrations of glycerol, and observations made of the resulting changes in motility of the spermatozoa.

2. These observations indicated that the damage suffered by the spermatozoa during freezing and thawing was caused by their exposure to excessive concentrations of salt when water was removed as ice.

3. The concentration of electrolyte above which irreversible damage occurred was characteristic for the spermatozoa of each species tested. The protective action of glycerol was due to its ability to prevent the salt concentration rising above this level.

We are indebted to Dr A. S. Parkes, F.R.S., for his interest and advice. Our best thanks are due to Dr Audrey U. Smith who made freely available the results of her wide experience in freezing living cells. We should also like to express our thanks to Mr J. H. S. Blaxter, of the Marine Laboratory, Wood Street, Torry, Aberdeen, who supplied the herring gonads.

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A Study of the Metabolism of Phosphorus in Mammalian Red Cells

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(*Received* 24 *June* 1954)

It has been well established that orthophosphate containing radioactive phosphorus ([^{32}P-]orthophosphate) present in the plasma enters the mammalian red cell by way of an exchange process without a net increase in the phosphate concentration within the cell (Hahn & Hevesy, 1941; Gourley, 1952.) The radioactive phosphorus thus taken up by the cell is present within the cell not only as orthophosphate but also in the form of organophosphate esters. One can therefore visualize two aspects of the phosphate metabolism of the red cell: (1) the extracellular–intracellular exchange process involving the transport of [^{32}P]orthophosphate across the cell membrane, and (2) the intracellular partition of phosphorus amongst orthophosphate and various organic phosphate esters.

This paper will deal with the intracellular partition of phosphorus as studied by means of the addition of [^{32}P]orthophosphate to the extracellular medium. It will be shown that the relationship of the relative specific activities (R.S.A.) of the intracellular phosphate esters to each other and to orthophosphate suggests that (*a*) orthophosphate enters glycolysis by way of the glyceraldehyde 3-phosphate dehydrogenase reaction, and (*b*) that the intracellular inorganic phosphate fraction is not the main source of the phosphorus incorporated into the ester phosphate.

In order to lend further support to the foregoing conclusions, measurement of the rate of disappear-

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ance of extracellular [^{32}P]orthophosphate and estimations of [^{32}P]distribution among phosphate ester components of the stroma were made.

METHODS

Between 0·05 and 0·15 ml. of 0·0007 N-$NaH_2{}^{32}PO_4$ (depending on the specific activity of the material) was added to 10 ml. of fresh, heparinized blood. In experiments lasting up to 6 hr. no glucose was added and no precautions were taken to ensure sterility, but in those lasting for longer periods sufficient glucose was added to raise the initial plasma concentration to about 400 mg./100 ml. and aseptic techniques were used. Incubation at 37·5° was carried out in siliconized glassware in order to minimize frothing. The blood was kept mixed and at nearly constant pH by bubbling a gas mixture containing 95% O_2 and 5% CO_2 slowly through a fine-bored glass tube.

Measurements of pH were made periodically on plasma samples of the incubating blood with a glass electrode. Over the course of 6 hr. no pH change was observed; over the course of 24 hr. the pH fell from a mean of 7·8 to 7·5.

At varying times suitable volumes of blood were removed and centrifuged at 2500 *g* for 15 min. The plasma was pipetted off and kept, and the cells washed first in an isotonic phosphate–saline solution (90 ml. 0·85% NaCl + 10 ml. 0·1 M-Na_2HPO_4) and secondly in isotonic saline, centrifuging being carried out at the same speed and for the same time at each washing. The 'buffy coat' was removed from the washed packed cells, which were finally frozen and kept in this state until further treatment.

The cells were then thawed and haemolysed by adding twice the volume of distilled water containing a trace of saponin, and 0·4 ml. of 0·2 M acetate buffer, pH 4·2. The haemolysate was spun in polyethylene tubes in a high-speed angle centrifuge (Servall) at 15 000 *g* for 20 min., the entire operation being performed in a cold room at 4°. The supernatant solution was carefully removed from the stroma and the latter washed twice more with the same acetate buffer; centrifuging was carried out as before. The stroma thus prepared was almost white. Both haemolysate and stroma were frozen, and on subsequent thawing 10% trichloroacetic acid (TCA) extracts were made by adding 0·1 vol. of a 100% (w/v) TCA solution to the haemolysate, and a known volume of 10% (w/v) solution of TCA to the stroma.

Chromatography. Phosphate esters were separated in the manner described by Caldwell (1953). The TCA extract (0·2 ml.) was dried on Whatman no. 4 paper (which had been washed previously with 2 N acetic acid) over an area 1 cm. or less in diameter, the whole process being carried out in a current of warm air. The chromatograms were run in *n*-propanol (60 ml.)–conc. NH_3 (30 ml.)–water (10 ml.) for about 16 hr. and *tert.*-butanol (80 ml.)–water (20 ml.)–picric acid (4 g.) for 5–6 hr. The esters could be located readily with a surface-scanning counter, and this method was adopted throughout. The following compounds were separated: adenosine triphosphate (ATP), adenosine diphosphate (ADP), 2:3-diphosphoglyceric acid (2:3-DPG), fructose 1:6-diphosphate, inorganic phosphate. The relative R_F values of these compounds are dealt with in detail in the papers of Hanes & Isherwood (1949) and Caldwell (1953).

Radioactivity assays. A standard area around each compound was cut out and the activity assayed with a thin-window Geiger–Müller counter. A similar area of paper known to be devoid of phosphate esters was assayed as background. The activity of the compounds was such as to give counting rates of 1000–3000 counts/min. Counting was continued for a sufficient length of time to give a statistical error of less than 3%. The radioactivity of the extracellular fluid was assayed by drying 0·05 ml. of the fluid on to a small area of filter paper and counting this in the same way.

Counts were also made occasionally on the supernatant fluid after the second washing of the cells, and it was found that practically all the radioactivity had been removed from the extracellular fluid; the activity was reduced to less than 0·5% of the original. Specific activities were corrected to a standard addition of 10^5 counts/min./ml. blood.

Chemical analyses. The area of paper containing each compound was ashed and oxidized with M-K_2SO_4 and conc. H_2SO_4 as described by Caldwell (1953), and orthophosphate was determined using a Kromatrol photoelectric colorimeter (Will Corp., Rochester, N.Y.) by the method of Berenblum & Chain (1938). For the haemolysates this method with 10 ml. of *iso*butanol was used. For the stroma

Table 1. *Reproducibility of findings on duplicate samples of blood*

R.S.A. = counts/min./μg. P.

	Sample *A*		Sample *B*	
Expt. 1	μg. P/ml. packed cells	R.S.A. at 2 hr.	μg. P/ml. packed cells	R.S.A. at 2 hr.
2:3-DPG	150	229	129	220
Inorganic phosphate	55·3	150	64·4	137
ATP	30·1	172	37·5	184
ADP	16·5	94	20·5	101
Fructose 1:6-diphosphate	15·0	67	12·0	75
	Sample *A*		Sample *B*	
Expt. 2	μg. P/ml. packed cells	R.S.A. at 4 hr.	μg. P/ml. packed cells	R.S.A. at 4 hr.
2:3-DPG	172	430	155	395
Inorganic phosphate	44·3	400	45·0	370
ATP	43·1	293	38·0	320
ADP	16·5	230	17·0	215
Fructose 1:6-diphosphate	11·1	100	9·3	115

the micromethod was used and separations were carried out in a small test tube; the final volume for colorimetry was 1·0 ml. As sodium arsenate was added during some of the experiments, separate tests were made to establish that this substance would not interfere with the colour produced in these analyses.

Reproducibility of methods. As interpretations of our results depend ultimately upon the reproducibility of the methods, tests were done on duplicate samples of the same blood incubated separately after the addition of the radioactive orthophosphate. It will be seen from Table 1 that the results based on chemical as well as on a combination of chemical and radioactivity determinations are reproducible within limits of 10–15%.

RESULTS

Phosphate compounds occurring in mammalian red blood cells. Many analyses of these compounds have been made by other workers, but with few exceptions the methods used are open to criticism, since they depend on salt precipitation and differential hydrolysis for the separation and characterization of each compound. The fractionation of cell extracts by means of the different solubility of the salts of phosphate compounds involves a number of errors principally due to coprecipitation and incomplete precipitation. Differential hydrolysis as a method for characterization of phosphate esters is particularly treacherous in red cell studies, since it has been shown that such criteria as the 7-min. hydrolysable phosphorus content are most inadequate (Mányai & Székely, 1954). At best such methods measure both ATP and ADP, and a small amount of hydrolysis of 2:3-DPG would introduce serious errors.

Thus such methods might give some guide as to the distribution of phosphate within the cell, but it is unlikely that they would achieve the unequivocal separation obtained by chromatography. Recently such separations have been made by Fleckenstein & Gerlach (1953), who used paper chromatography with solvents different from ours, and by Bartlett, Savage, Hughes & Marlow (1953), who used ion-exchange methods.

Precise separations can be achieved by both means and the results obtained differ to a considerable extent from those of earlier workers. The values obtained by us agree well with those obtained by the two authors mentioned above and are shown in Table 2. In this table also are shown the values obtained for other mammalian erythrocytes.

Extracellular–intracellular exchange of ^{32}P

Phosphate exchange in normal human red cells. Experiments were performed with a number of blood samples from different donors, and with samples from one donor at different dates. The rate of exchange of ^{32}P between plasma and cells was estimated by plotting the $\log_{10}$ of the residual ^{32}P activity of the plasma against time. A straight line is obtained if the points taken are within the first 4 hr. of incubation. After this time the intracellular and extracellular ^{32}P concentrations approach equilibrium and the slope flattens. These findings are in agreement with the earlier work of Gourley & Matschiner (1953), and indicate that ^{32}P transfer is a first-order process. A composite constant for the exchange process was calculated using the relation

$$K = 1/t \ln (C_0/C_t),$$

where t = hours, C_0 = extracellular radioactive phosphorus concentration at the beginning of the experiment and C_t = extracellular radioactive phosphorus concentration at time t. The constants thus obtained have been corrected to a standard haematocrit of 40%. Other authors have reported wide variations in the rate of ^{32}P exchange (Pertzoff & Gemmill, 1949), but it will be seen from Fig. 1 that the rates are closely reproducible if the conditions are constant. The rates of exchange in a single individual observed at a number of different times over the course of 6 months were also found to be constant (Fig. 1).

Table 2. *Chemical constitution, diameter/thickness ratios* (D/Th), *and rates of glycolysis of mammalian red cells*

	As μg. P/ml. of packed cells						
	ATP	ADP	2:3-DPG	Inorganic phosphate	K*	D/Th†	Glycolytic‡ rate
Man	25–80	12–25	120–180	35–64	110	3·7	15§
Dog	14–25	10–20	180–205	35–45	5	3·8	16§
Rabbit	20–35	15–25	180–240	50–70	90	3·7	18§
Cat	12–18	5–12	50–60	20–25	5	2·8	12·5‖
Ox	25–30	15–20	—	20–35	30	2·7	3·7§
Goat	20–25	12–18	—	17–26	18	2·0	8·6§

* As μmol. K/ml. packed cells. Data from Kerr & Daoud (1935).
† Data from Wintrobe (1952).
‡ As mg. glucose/hr./100 ml. of whole blood.
§ Data from Izzo (1949).
‖ Data from Hsu (1935).

Temperature dependence of phosphate exchange. The exchange of ^{32}P at three temperatures, 37·5°, 28·0°, 15·5°, all ±0·05°, was measured. At the lowest temperature there is only very slow penetration of ^{32}P. A composite diagram showing the $\log_{10}$ of the labelled phosphorus of the residual plasma plotted against time at the three temperatures is shown in Fig. 2. On the same figure is shown also the $\log_{10}$ of the transfer constant plotted against the reciprocal of its absolute temperature. From these values the activation energy of phosphate transfer has been calculated to be 19800 cal./mol. This value is somewhat higher than 16000 cal./mol. found by Gourley & Gemmill (1950), but both these values are 3–4 times that which would be expected for an exchange process resulting only from passive diffusion.

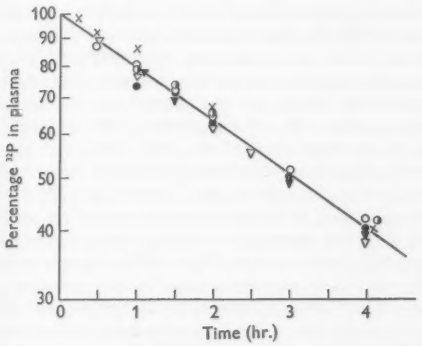

Fig. 1. Time course of ^{32}P exchange in different samples of blood and in one sample of blood on three different occasions. ●, TAJP, Dec. 1953; ▼, TAJP, Mar. 1954; ▽, TAJP, Feb. 1954; ○, LEY, Feb. 1954; ◑, LEY, Feb. 1954; ×, GM, Mar. 1954.

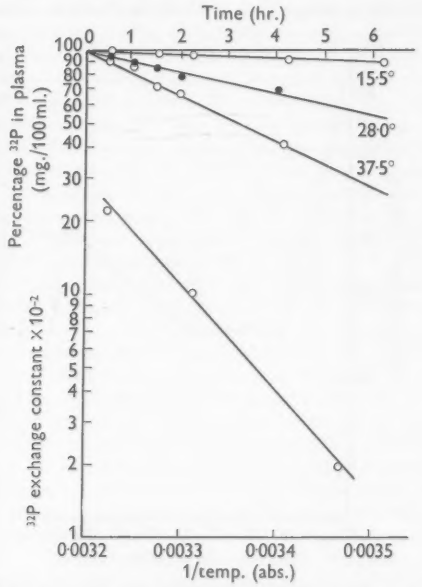

Fig. 2. Composite figure showing rates of ^{32}P exchange at three temperatures as indicated, with relationship of rate constants to the reciprocal of the absolute temperatures (lower figure).

At 3° the rate of exchange is negligible except during the first hour when a small but measurable exchange occurs. It is unlikely that this exchange is a result of inadequate temperature equilibration, since the blood was kept at 3° for 20 min. before the addition of [^{32}P]orthophosphate. Its short duration argues against the assumption that it constitutes a diffusion effect. It is conceivable that the effect (also observed by Gourley, 1952) is related to an adsorption process essentially physical in nature and unrelated to a metabolic transport phenomenon.

Effects of inhibitors. Either sodium fluoride or iodoacetate was added to blood after 2 hr. incubation at 37·5° (Fig. 3). In neither instance is the observed effect immediate, but occurs over the course of about 1 hr., producing in the case of iodoacetate a cessation, and in the case of sodium fluoride only a slowing of phosphate exchange. A similar latent interval was observed by Harris & Maizels (1951) on the effect of these inhibitors on sodium exchange. With both substances the plasma inorganic phosphorus rose by about 10 μg./ml., indicating that there was an escape of phosphorus from the cells.

Effects of different substrates

Adenosine. Dische (1938) observed that red cell haemolysates incubated with adenosine in the presence of sodium fluoride would take up phosphate and form ribose 5-phosphate. Later, Dische (1951) showed that this was converted into fructose

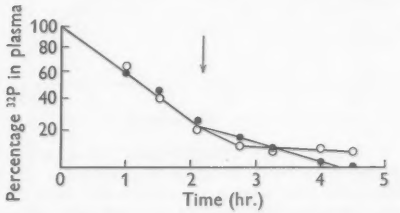

Fig. 3. The effect of the addition of sodium fluoride (1×10^{-3} M) and sodium iodoacetate (1×10^{-3} M) on phosphate exchange. Inhibitors added at time indicated by arrow. ○, blood with sodium iodoacetate; ●, blood with sodium fluoride.

1:6-diphosphate, hexose monophosphate, and triose phosphate.

As it was desirable to determine whether intact red cells could utilize adenosine, blood was taken from a donor early in the morning when his blood sugar was at a fasting level, and was incubated alone for 6 hr. At the end of this time the residual glucose concentration had fallen to 3 mg./100 ml. The blood was then divided into two parts to each of which [^{32}P]orthophosphate was added. To one part an adenosine solution in 0·9% saline was added to give a final adenosine concentration of 0·005 M, and to the other a volume of isotonic saline equivalent to that of the adenosine solution. Incubation followed as usual and the results are shown in Fig. 4. The early fall in plasma ^{32}P-activity in the control sample was presumably due to the presence of the small amount of residual glucose. The [^{32}P] exchange is well sustained in the presence of adenosine with a rate constant (0·26 per hr.) slightly higher than that for glucose.

In order to show that the effect of adenosine resulted from the utilization of its ribose moiety, i.e. that this effect did not require the presence of a specific purine and that the primary reaction in the utilization of the *N*-riboside involved a phosphorylase, a similar experiment was performed with guanosine, and with adenosine and sodium arsenate. The addition of arsenate may be expected to result in the arsenolysis of adenosine or of glyceraldehyde 3-phosphate thus blocking phosphate exchange.

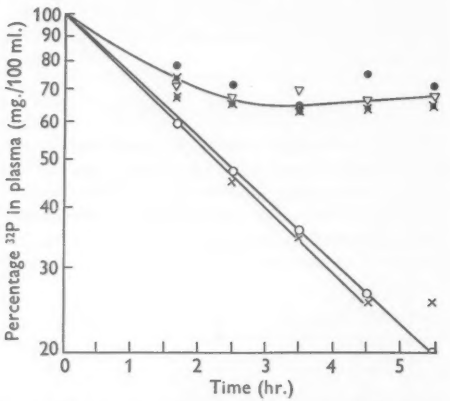

Fig. 4. Time course of ^{32}P-exchange in red cells after prior incubation for 6 hr., following the addition of different substrates. The plasma glucose at the onset of the experiment was 4 mg./100 ml. ×, cells with adenosine (5×10^{-3} M); ○, cells with guanosine (5×10^{-3} M); ✕, cells with adenosine (5×10^{-3} M) + sodium arsenate (5×10^{-3} M); ●, cells with glucose (200 mg./100 ml.) + sodium arsenate (5×10^{-3} M); ▽, cells with no added substrate.

Fig. 4 shows the inhibiting effect of arsenate in the presence of adenosine or additional glucose. Incubation of blood which contained adenosine as well as physiological amounts of glucose did not alter the rate constant, which remained close to that found for glucose alone.

One further experiment was performed with adenosine, the results of which will help to elucidate some of the findings described later in this paper. Whole blood was incubated with [^{32}P]orthophosphate and additional glucose, samples being taken after 6 and 24 hr. At the latter time the plasma glucose was still 150 mg./100 ml. The remaining blood was then divided into two parts, to one of which adenosine was added, and to the other 0·9% saline. The changes in the extracellular fluid are shown in Fig. 5, where it will be seen that in the first 6 hr. the phosphate exchange was normal. At some time between 6 and 24 hr. ^{32}P escapes from the cells and this continues despite the presence of glucose in amounts adequate for glycolysis. The addition of adenosine however leads to a resumption of ^{32}P-uptake until a new equilibrium is attained at a lower level of ^{32}P-concentration.

Glutathione. The human red cell contains significant amounts of this substance, about 700 mg./l. of cells (Maizels, 1939). Addition of glutathione to red cells leads to an increase in the oxygen consumption of mature mammalian red cells and affects cation

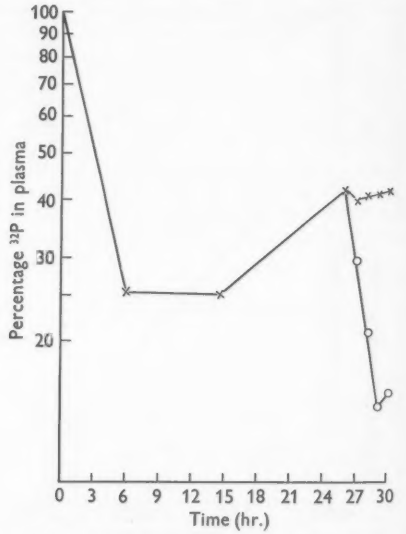

Fig. 5. Time course of ^{32}P-exchange over 30 hr. showing the effect of the addition of adenosine (5×10^{-3} M) at 27 hr. The plasma glucose concentration immediately before the addition of adenosine was 200 mg./100 ml. ○, blood with adenosine; ×, blood with no additional substrate.

exchange (Bruns, 1950). The addition of glutathione in final concentrations of 0·06 M together with 0·01 M-$MgSO_4$ was tried in our system, but no alteration in ^{32}P-exchange was observed.

Observations on other mammalian cells. Blood from five other mammals was obtained in order to assess any correlation which might exist between metabolism, chemical composition and shape (Table 2). Thus the ox and goat cell contain practically no 2:3-DPG, low concentrations of potassium, and are smaller in diameter and thicker than the human cell (Kerr, 1937; Fleckenstein & Gerlach, 1953; Wintrobe, 1952). The cells of both these species exhibit a low rate of phosphate exchange, as shown in Table 3, together with rate constants for dog, rabbit and cat red cells. Dog cells approximate closely to human cells in shape but have a greater content of 2:3-DPG and a very much lower content of potassium. Rabbit cells also have more 2:3-DPG and a similar shape, but their potassium content lies between that of dog and human cells. Dog, rabbit and human cells have approximately the same rate constants. The cat cells were obtained from an animal under barbiturate anaesthesia, and this may have played some part in the low rate of exchange that these cells showed (Pertzoff & Gemmill, 1949).

Intracellular phosphate partition

This aspect of the cell's phosphate metabolism has been studied by estimating the relative specific activity (R.S.A.) and total intracellular concentration of the various phosphate intermediates that have been separated. Provided that the cells maintain a steady state, an interpretation can be made of the rates at which glycolytic reactions are proceeding within the cell as well as in the stroma.

An analysis of the R.S.A. of a group of intermediates with respect to time is shown in Fig. 6. Up to 6 hr. the most active fraction is 2:3-DPG which bears a precursor relationship to ATP. With the exception of the first hour these compounds exhibit a considerably greater activity than orthophosphate. Gourley (1952) found that the isotope concentration of ATP and 2:3-DPG was higher than that of orthophosphate, but that contrary to our findings, ATP had a higher R.S.A. than 2:3-DPG. It will be pointed out later in this paper, as has been done elsewhere (Prankerd & Altman, 1954), that the relationships found here are what might be expected on the basis of our knowledge concerning the entry of inorganic phosphate into glycolysis at the triose phosphate dehydrogenase level. At equilibrium the R.S.A. of ATP, 2:3-DPG and inorganic phosphate are approximately equal, but those of ADP and fructose 1:6-diphosphate are appreciably lower.

It should be pointed out here that the R.S.A. of all compounds containing more than one phosphorus atom per molecule constitute merely the average R.S.A. of all phosphorus atoms present. No attempt has been made to differentiate between the R.S.A. of phosphorus atoms having different rates of turnover. Thus our data for the R.S.A. of ATP cannot be compared directly with those reported by Gourley (1952). If it is assumed that the adenylic acid phosphorus atom has an activity of approximately one-fourth or less of that of the two terminal phosphorus atoms during the first few hours of incubation, then an appropriate dilution factor would have to be applied to our R.S.A. values, raising them to a somewhat higher level. The R.S.A. of 2:3-DPG also constitutes an average of two phosphorus atoms differing widely in their R.S.A. Independent estimates have revealed that the ^{32}P-activity of 3-phosphoglyceric acid is extremely low, indicating that the radioactivity contribution of the phosphate group esterifying the hydroxyl group attached to C-3 of 3-phosphoglyceric acid is small. This would mean that R.S.A. of the other phosphate group is approximately 40 % higher than indicated. In view of these considerations a precursor relationship undoubtedly exists between the labile terminal phosphorus atoms of ATP and the 2-phosphate of 2:3-DPG. If similar considerations

Table 3. *Rate constants (per hour) for* ^{32}P*-exchange in various mammalian red cells*

Human cells incubated with adenosine and glucose, the others with glucose.

Man (+adenosine)	0·26	Cat	0·11
Man (+glucose)	0·24±0·01	Ox	0·07
Dog	0·23±0·01	Goat	0·07
Rabbit	0·21±0·01		

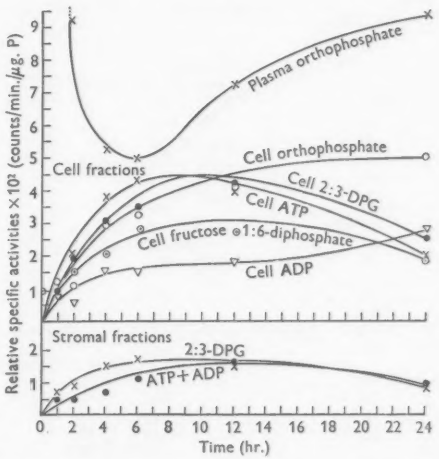

Fig. 6. Time course of ^{32}P-exchange, intracellular phosphate partition, and stromal phosphate fractions during 24 hr. after the addition of ^{32}P.

are applied to the data of Gourley (1952), such a precursor relationship is also present, although this was overlooked because of the failure to take into account the fact that the R.S.A. of 2:3-DPG consitutes the average isotope concentration of two phosphorus atoms of different activity.

When cells are incubated for periods longer than 6 hr. there is a fall in the R.S.A. of ATP and 2:3-DPG with a rise in that of orthophosphate, indicating that the system is no longer maintaining its original equilibrium. Some haemolysis occurs and the R.S.A. of plasma orthophosphate rises. In addition to the fall in ^{32}P-activity of the intracellular esters, there is also a fall in the total quantities of these esters, particularly 2:3-DPG, and to a less extent ATP; the amount and R.S.A. of the intracellular orthophosphate rises (Table 6). It is difficult to see how this fall in the R.S.A. values is brought about and possible mechanisms will be discussed later.

In Fig. 6 the R.S.A. of three compounds derived from the stroma are also shown. The amounts of these compounds are small and for this reason it has been impossible to separate ATP from ADP. The R.S.A. in the stromal fraction are much smaller than their intracellular counterparts.

Temperature dependence. The changes observed are depicted in Table 4. The turnover of compounds labelled with ^{32}P is greatly reduced at 15°, paralleling the findings for the exchange process. It appears that at low temperatures the turnover of ^{32}P in the 2:3-DPG fraction is reduced to such an extent that its activity no longer bears the precursor relationship that was observed at 37·5°.

Effects of inhibitors. It will be seen (Table 5) that sodium fluoride leads to a fall in the total amount of ATP with a more marked fall in its R.S.A., whereas 2:3-DPG tends to increase in amount with a slow but parallel rise in its R.S.A. This presumably occurred because phosphate exchange did not cease but was merely slowed; similar findings were observed by Rapoport & Guest (1939). Iodoacetate leads to a decrease in both amount and specific activity of 2:3-DPG and ATP. In both instances the intracellular orthophosphate rises in amount and activity coincident with the fall in the ester fractions. Considering the well known locus of action of these inhibitors, the changes are what might be expected.

Effect of different substrates

Adenosine. The intracellular and stromal fractions obtained in the experiments described earlier are summarized in Tables 6 and 7. The data in Table 6 demonstrate that adenosine, when added 24 hr. after incubation, brings about a regeneration of the 2:3-DPG and ATP contents of the cells and an increased incorporation of ^{32}P into these compounds. In spite of the fact that the plasma glucose concentration was still 200 mg./100 ml. after 24 hr. of incubation, analyses of those incubated portions

Table 4. *Effect of temperature on intracellular ^{32}P-turnover*

R.S.A. = counts/min./μg. P.

Temp. (°)		R.S.A. at varying times after addition of ^{32}P			
		0·5 hr.	2 hr.	4 hr.	6 hr.
37·5	2:3-DPG	85	222	486	375
	Inorganic phosphate	100	195	362	390
	ATP	45	168	400	370
	ADP	20	84	150	204
28·0	2:3-DPG	48	53	101	—
	Inorganic phosphate	119	179	168	—
	ATP	34	116	132	—
	ADP	25	56	68	—
15·0	2:3-DPG	3·5	5·1	—	13
	Inorganic phosphate	18	29	—	65
	ATP	14·5	16	—	29
	ADP	—	—	—	—

Table 5. *Effect of addition of sodium fluoride or iodoacetate after 2 hr. previous incubation with ^{32}P*

R.S.A. = counts/min./μg. P. Final concentrations: sodium fluoride = 0·01 M, iodoacetate = 0·03 M.

	Control		Sodium fluoride		Iodoacetate	
	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
2:3-DPG	160	220	179	380	123	196
Inorganic phosphate	55	210	84	290	85	380
ATP	29	120	15	40	10	20
ADP	15	80	29	85	21	75

Table 6. *The effect of the addition of adenosine to blood incubated for 25 hr. with [^{32}P]orthophosphate and an excess of glucose*

A = 25 hr. incubation before the addition of adenosine; *B* = following the addition of adenosine (0·005 M) after 25 hr. incubation; *C* = without the addition of any extra substrate at 25 hr. Plasma glucose after 25 hr. incubation = 200 mg./100 ml. R.S.A. = counts/min./μg. P.

		Hours of incubation with ^{32}P					
		6 hr.		12 hr.		25 hr.	
Expt.		μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
A	2:3-DPG	148	330	84	356	39	240
	Inorganic phosphate	35	240	32	685	63	400
	ATP	58	210	35	230	25	200
	ADP	21	130	27	150	12	146
		26 hr.		30 hr.			
B	2:3-DPG	73	455	79·5	432		
	Inorganic phosphate	45	340	51	276		
	ATP	32	195	58·5	293		
	ADP	12	63	25·5	153		
C	2:3-DPG	30	232	45	184		
	Inorganic phosphate	66	326	61·5	440		
	ATP	30	310	27	124		
	ADP	16·5	180	23	268		

Table 7. *The effect of the addition of ribosides with and without arsenate, and glucose with and without arsenate, on blood which had been incubated for 6 hr. before addition of ^{32}P*

^{32}P and substrates all added at 0 hr. *A* = Adenosine (0·005 M) alone; *B* = adenosine (0·005 M) + Na_2HAsO_4 (0·004 M); *C* = glucose (150 mg./100 ml.) + Na_2HAsO_4 (0·004 M); *D* = guanosine (0·005 M) alone; *E* = no substrate or inhibitor added (molar concentrations of the substrates refer to plasma rather than whole blood). Plasma glucose at 0 hr. (i.e. after 6 hr. of incubation) before addition of substance = 4 mg./100 ml. R.S.A. (counts/min./μg. P) in brackets denotes stromal activities.

		Hours of incubation with ^{32}P and substrates					
		2 hr.		4 hr.		6 hr.	
Expt.		μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.	μg. P/ml. packed cells	R.S.A.
A	2:3-DPG	116	195 (52)	127	426	135	370 (95)
	Inorganic phosphate	45	185	48	416	45	400
	ATP	48	170 (0)	46·5	215	57	340 (54)
	ADP	31	135	25	95	36	66
B	2:3-DPG	78	95 (15)	55·5	64	27	30 (0)
	Inorganic phosphate	66	256	81	314	90	310
	ATP	16·5	24 (0)	25	110	27	40 (0)
	ADP	9·0	50	21	26	20	30
C	2:3-DPG	48	190 (0)	46·5	180	26	61 (0)
	Inorganic phosphate	48	360	79·5	364	117	344
	ATP	18	29 (0)	20	31	30	73
	ADP	16·5	19	13	19	21	22
D	2:3-DPG	117	120 (40)	156	193	150	340 (106)
	Inorganic phosphate	46	113	84	226	66	390
	ATP	45	109 (10)	27	190	36	224 (74)
	ADP	21	91	22	100	30	86
E	2:3-DPG	92	55 (20)	—	—	33	42 (25)
	Inorganic phosphate	54	265	—	—	61·5	225
	ATP	40	123 (5)	—	—	24	32 (10)
	ADP	21	61	—	—	16·5	40

of blood to which no adenosine was added after 24 hr. showed a progressive fall in 2:3-DPG with a decrease in the R.S.A. of this ester and of ATP. Such a finding suggests that although on incubation *in vitro* the red cell loses its ability to metabolize glucose after some 24 hr., the utilization of adenosine continues at a rate comparable to that prevailing during the first 4 hr. of incubation.

In Table 7 are shown the intracellular counterparts of the experiments in which the ribosides were added after all of the glucose had been metabolized during the preceding 6 hr. incubation period. In the absence of added ribosides, the concentration of 2:3-DPG decreased rapidly during the second 6 hr. coincident with a lesser decrease in ATP. At the same time the incorporation of ^{32}P into these esters was minimal. The effect of adenosine or guanosine in promoting the uptake of ^{32}P into both esters is dramatic and is accompanied by a net increase in 2:3-DPG. The addition of glucose in the presence of arsenate produces a different pattern of intracellular partition to that of adenosine in the presence of arsenate. In the former case the flow of ^{32}P into the 2:3-DPG fraction proceeds at a significantly greater rate and the R.S.A. of this fraction and also of ATP is higher than in the latter case. This difference may be attributed to the greater extent to which arsenolysis proceeds in the case of adenosine as compared with glyceraldehyde 3-phosphate. Similar changes were found in the stromal fractions.

Other mammalian cells. In the dog and rabbit the intracellular relationships are unlike those found in man. In the red cells of these animals the precursor relationship between 2:3-DPG and ATP is present, but the activity of orthophosphate exceeds that of either of these two esters. In ox and goat cells where no 2:3-DPG is present orthophosphate activity exceeds that of ATP. These changes are in part a result of greater orthophosphate activity and in part a result of diminished ester activity.

DISCUSSION

The uptake of orthophosphate by the cell and its transport across the cell membrane is thought to depend primarily upon glycolysis, particularly at the level of the oxidative phosphorylation of glyceraldehyde 3-phosphate. Such a concept points to an intimate relationship between phosphate uptake and glycolysis. It should be stated here that this does not imply the existence of a direct relationship between phosphate uptake and glucose transport, since the red cell membrane is peculiarly permeable to free glucose. The inhibition of phosphate uptake by inhibitors of glycolysis such as sodium fluoride and iodoacetate, as well as the finding that exhaustion of the cell's glucose supply leads to a cessation of phosphate exchange lend further support to the view that phosphate uptake and glycolysis are interdependent processes.

In order to elucidate the relationship between these two processes in the mammalian red cell, the following reactions deserve consideration:

If glycolysis is directly concerned with the uptake of phosphate by the red cell, a reaction into which

$$\text{glyceraldehyde 3-phosphate} + DPN^+ + \text{enzyme} \rightleftharpoons \text{3-phosphoglyceryl–enzyme} + DPNH + H^+. \quad (1)$$

$$\text{3-phosphoglyceryl–enzyme} + [^{32}P]\text{orthophosphate} \rightleftharpoons [1\text{-}^{32}P]\text{1:3-diphosphoglyceric acid} + \text{enzyme} \quad (2)$$

$$[1\text{-}^{32}P]\text{1:3-diphosphoglyceric acid} + ADP \xrightleftharpoons{\text{phosphoglycerokinase}} \text{3-phosphoglyceric acid} + [\textit{terminal}\text{-}^{32}P]ATP. \quad (3)$$

$$[1\text{-}^{32}P]\text{1:3-diphosphoglyceric acid} \xrightleftharpoons{\text{phosphoglyceromutase}} [2\text{-}^{32}P]\text{2:3-diphosphoglyceric acid.} \quad (4)$$

Rapoport & Luebering (1950)

orthophosphate can enter directly should be involved. Such a reaction is (2) which must be preceded by the oxidative step as represented by reaction (1) (Segal & Boyer, 1953; Oesper, 1954; Harting & Velick, 1954). From reaction (4) it is evident that 1:3-DPG is in equilibrium with 2:3-DPG, which is present in human red cells in amounts accounting for about half the total phosphorus content of the cell. Reaction (4) shows that the phosphate group in position 1 of 1:3-DPG, and in position 2 of 2:3-DPG are equivalent and that in accordance with reaction (3), the labelled phosphate group of 1:3-DPG is a precursor of the labile terminal phosphate group of ATP. From this sequence of events it may be predicted that 2:3-DPG should bear the same relationship as 1:3-DPG to ATP. Such a relationship has been verified by our experiments, and may, as we have seen, be extrapolated also from the results obtained by Gourley (1952), although this author had reached the opposite conclusion.

As to the site at which organic phosphate is linked to red cell glycolysis, our data, as well as Gourley's, suggest that phosphate is not participating in reaction (2) at the intracellular level, because the R.S.A. of intracellular orthophosphate is lower than that of both 2:3-DPG and ATP (except during the first hour). These findings indicate that

the intracellular orthophosphate may be derived from ATP as has been suggested by Rothstein (1954) for the yeast cell. These findings were not observed in cells of other species such as the dog and rabbit, suggesting that in these animals orthophosphate must be derived from other sources as well as ATP. It is possible that the diminished ATP activity in these cells is a reflexion of their low potassium contents, but further analyses are required to clarify these points.

On the premise that orthophosphate does not enter reaction (2) at the intracellular level, one must search for other sites at which it might participate in the reaction. Rothstein's suggestion that in the yeast cell phosphorylation is carried out on the cell surface seems most pertinent, and if this view can be extended to the red cell, keeping in mind that this cell differs markedly from the yeast cell with respect to glucose transport, then our findings related to the stromal fractions assume significance.

We have established that certain intermediates of glycolysis are present in the stromal fraction and that these incorporate ^{32}P derived from plasma orthophosphate. Although this might be thought to result from contamination with intracellular material, there are two points that make it unlikely. First, the stroma preparation was washed so thoroughly that any residual compounds must have been tightly bound; the completeness of the washing was confirmed by the absence of activity in the supernatant washings. Secondly, the R.S.A. values of the stromal intermediates do not parallel those found intracellularly, as they should do were they contaminants. It seems probable that these stromal intermediates indicate glycolysis having a specific function such as either the directed transport of phosphate, electrolytes, or glucose, or the supply of energy for the maintenance of the unusual biconcave shape of the human red cell. It was the last possibility that prompted us to investigate the stroma of the ox and goat cells, which are spherical. Virtually no activity could be detected in the stromal fractions from these species. More evidence is required before one can accept the hypothesis that glycolysis in the stroma is related to the cell's shape, but it may not be too remote to visualize the red cell envelope as possessing certain contractile properties, dependent upon a continuous regeneration of ATP.

Additional support for our concept of the active transport of phosphate into the red cell is gained from the magnitude of the energy of activation of this process, namely 19800 cal./mol. In general the activation energy of a passive diffusion process would involve only about 4000–5000 cal./mol. Our finding that phosphate exchange virtually ceases at 15° emphasizes the sensitivity of this process to a fall in temperature. This observation, together with those of Gourley (1952) and Halpern (1936), conflict with that of Hahn & Hevesy (1941), who found appreciable phosphate exchange at 0°. Probably a low order of passive phosphate diffusion occurs simultaneously with active transport, for Gourley & Matschiner (1953) found two exponential processes involved in phosphate exchange, signifying a more rapid process in the first half hour than at later times, and we have found a small degree of phosphate penetration into the cell during the first hour at 3° which did not increase subsequently.

Although probably of minor physiological importance there exists another mode by which phosphate may enter the red cell. The work of Dische (1938, 1951) with adenosine and guanosine made it clear that orthophosphate may be esterified by phosphorolysis of purine ribosides giving rise to the formation of ribose phosphates and thus to triose and hexose phosphates. The participation of adenosine and guanosine in mediating phosphate transport is well shown in the experiments in which ^{32}P-equilibrium is restored by the addition of the ribosides after 24 hr. incubation without them. After this period adenosine appears to be more effective in restoring the equilibrium than glucose, and it occurs at a time when the intracellular ATP has fallen to a level which is probably insufficient for the phosphorylation of glucose. Phosphorolysis of purine ribosides does not require ATP. These circumstances resemble those prevailing in Dische's experiments where ATP regeneration was blocked by the addition of sodium fluoride, and glycolysis inhibited without interfering with the phosphorolysis of the ribosides. As far as our experiments with these substances are concerned their phosphorolytic cleavage is thought to have two important consequences, (*a*) replenishing the depleted stores of 2:3-DPG by way of the formation of glyceraldehyde 3-phosphate formed from the enzymic breakdown of ribose 5-phosphate, and (*b*) forming fructose 1:6-diphosphate and hexose monophosphate in the manner described by Dische (1938, 1951). It thus appears that adenosine is more effective potentially than glucose as a substrate for glyceraldehyde 3-phosphate formation in the red cell, because the investment of energy, is smaller in the case of adenosine which does not require ATP for the phosphorylation of its pentose moiety. These concepts are pertinent to the interesting work of Gabrio, Stevens & Finch (1954) and Gabrio & Finch (1954) who showed an increased viability of red cells stored at 4° in acid–citrate–dextrose solution when purine ribosides were added. The role of these substances in phosphate exchange suggested by Dische's and our experiments could well explain Gabrio's observations.

A word of explanation is required for the falling R.S.A. of the intracellular 2:3-DPG and ATP between

6 and 24 hours. Such a fall would require the re-exchange of ^{31}P for ^{32}P in these esters. Since the compounds separated had all reached equilibrium by this time, such ^{31}P would have to come from an unequilibrated source. One such source might be the phospholipid fraction.

Explanation is also required for the rising extra- and intracellular orthophosphate activity after 24 hr. The former would appear to be due largely to a loss of orthophosphate from the red cells, since these cells resume phosphate exchange once a metabolizable substrate is added. Slight haemolysis and leucolysis also contribute to this rise in the R.S.A. of inorganic phosphate; any organic phosphates liberated in this way being broken down by the phosphatases of the plasma (Meister, 1947). No detailed examination of the number of leucocytes in the blood samples has been made, but it is unlikely that these cells contributed significantly to phosphate exchange in our system since the number of leucocytes present must have varied within the physiological range and yet the rates of phosphate exchange remained constant. This might appear surprising since leucocytes utilize glucose at a rate several times that of red cells (Hsu, 1935). However, anaerobic glycolysis by leucocytes may have been greatly inhibited, since the oxygen saturation of the blood during incubation may be expected to invoke the Pasteur effect. The rising intracellular orthophosphate in these experiments is most likely the result of the breakdown of adenosine diphosphate, and the action of phosphatases (Tsuboi & Hudson, 1953; Lennerstrand & Lennerstrand, 1940).

It is a provocative fact that some mammalian red cells contain great amounts of 2:3-DPG, whereas others contain almost none at all. Those cells which contain negligible amounts of 2:3-DPG also have a small diameter/thickness ratio (Table 2), and also have a low ATP/ADP ratio. One is tempted to associate these phenomena with the shape of the cell, that is, to postulate that spherocytosis is related to low energy stores within the cell. This possibility is supported by the low phosphate turnover in the esters of the stroma from those species with spheroidal red cells. 2:3-DPG is contained in most other cells of the body in minute quantities only, merely sufficient to fulfill the requirements for its coenzyme function in the conversion of 3-phosphoglycerate into 2-phosphoglycerate, a reaction catalysed by phosphoglyceromutase (Sutherland, Posternak & Cori, 1949). In this reaction 2:3-DPG is continually regenerated and is therefore only required in catalytic amounts. In the red cell it may be reasonable to regard 2:3-DPG as a potential energy source upon which the cell may draw for the regeneration of ATP by way of reaction (3).

If human red cells are incubated in their own plasma, the plasma glucose is used up in about 6 hr. Haemolysis does not begin for about 26 hr. If the cell is to continue to carry on its metabolic activities in the absence of glucose, it can only do so at the expense of its intracellular energy stores. This is borne out by the fact that 2:3-DPG continues to disappear at a rate faster than ATP during the 6–24 hr. incubation period. That the red cell may meet similar situations involving the exhaustion of extracellular substrate *in vivo* is possible if one considers the slow circulation through such a vascular organ as the human spleen where haemoconcentration may occur leading to a haematocrit as great as 80 % (Gibson *et al.* 1946). In addition to the large number of red cells per mole of available glucose, the large mass of generative lymphoid tissue will be competing for the same substrate. In such circumstances the red cell may be faced with 'relative starvation', the final effects of which will depend upon the duration of this deprivation. In a number of species there is a correlation between the size of their red cells, the cell's content of 2:3-DPG, and the cation gradients existing between the cells and plasma (Table 2). Small cells are less likely, for mechanical reasons, to stagnate in vascular organs (Whipple, 1941; Björkman, 1947; Young, Platzer, Ervin & Izzo, 1951) and the absence of high ionic gradients would lower the energy demands of such cells and, therefore, the necessary size of the storage depots.

Such considerations give a rational explanation of the biological variations of mammalian cells and may have some fundamental bearing on the understanding of the correct approach to red cell storage, and to the viability of the red cell in diseased states, a subject which will be discussed in a later paper.

SUMMARY

1. The exchange of ^{32}P between various mammalian red cells and their plasma has been studied, and the effects of temperature, inhibitors of glycolysis, and various substrates observed.

2. The dependence of ^{32}P-exchange upon glycolysis has been demonstrated and also the participation of purine ribosides in this process.

3. By means of paper chromatography of red cell extracts, the intracellular partition of ^{32}P has been studied, and a precursor relationship found between 2:3-diphosphoglyceric acid and adenosine triphosphate. The rationale of this has been discussed.

4. Glycolytic intermediates have been found in the stromal fractions of red cells and their possible significance considered.

5. The effects of temperature, inhibitors of glycolysis, and certain substrates on intracellular phosphate partition have also been investigated.

Adenosine has been found to form a substrate for regeneration of the essential energy stores of the red cell, even after prolonged incubation when it appears that glucose can no longer be utilized.

6. An attempt has been made to explain some of the biological variations in the red cells of certain mammalian species on a rational basis, and to intimate the importance of metabolism in the storage of red cells and their diseased states.

We wish to thank Mrs M. Murphy for helpful technical assistance. One of us (T. A. J. P.) wishes to thank Dr L. E. Young for his kindness in providing facilities for this research, and Professor M. Maizels, who initiated his interest in these problems. The authors are indebted to Professor Maizels, Drs A. Rothstein, K. Salomon, L. E. Young and E. J. Harris for helpful criticism and discussion in the preparation of this manuscript, and to Dr A. Dutton for advice on the statistical handling of the data. This paper is based in part on work performed under contract with the U.S. Atomic Energy Commission, at the University of Rochester, Atomic Energy Project.

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The Amino Acid Sequence in Bacitracin *A*

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(*Received* 30 *June* 1954)

Bacitracin *A* is the major constituent of a family of antibacterial polypeptides known as bacitracin (Craig, Gregory & Barry, 1949; Newton & Abraham, 1950). It has a molecular weight of about 1470 and has been reported to yield the following amino acids on hydrolysis: D-ornithine (Orn) (1), L-lysine (1), L-histidine (1), DL-aspartic acid (2), D-glutamic acid (1), L-cysteine (1), D-phenylalanine (1), L-leucine (1), L-isoleucine (2) (Craig, Weisiger, Hausmann & Harfenist, 1952; Craig, Hausmann & Weisiger, 1952, 1953; Newton & Abraham, 1953*a*,*b*). In addition, it yields a significant amount of a substance which runs slightly faster than isoleucine on columns of Dowex 50 (Craig, Hausmann & Weisiger, 1952). This substance has been detected independently by Porath (private communication)

and in this laboratory. Piez (1954) has suggested that it is D-*allo*isoleucine. If this is so, it appears to be formed in considerably less than one molecular proportion. However, in a publication that became available after the present work had been carried out, Craig, Hausmann & Weisiger (1954) state that they have been forced to the conclusion that bacitracin *A* contains three isoleucine residues instead of two. Suggestions have also been made that bacitracin *A* contains an uncharacterized component which is not an amino acid (Craig *et al.* 1953; Porath, 1953).

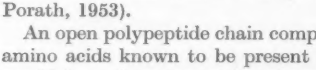
An open polypeptide chain composed only of the amino acids known to be present in the molecule would have four free carboxyl groups, three free amino groups, and a fourth basic group due to the glyoxaline ring of histidine. Bacitracin *A* was found to contain only two free carboxyl groups and only three free basic groups, the ε-amino group of the lysine residue showing no reaction with 1:2:4-fluorodinitrobenzene (Newton & Abraham, 1953*b*; Craig *et al.* 1953). Newton & Abraham (1953*b*) suggested that one of the masked carboxyl groups might be present as an amide, while the other might be engaged, directly or indirectly, in ring formation with the masked amino group of lysine.

Two of the three basic groups of bacitracin *A* were accounted for by the glyoxaline ring of histidine and the δ-amino group of ornithine. The third basic group titrated in the region expected of the α-amino group of an N-terminal amino acid residue (Newton & Abraham, 1953*b*) and apparently behaved like an amino group in the Van Slyke determination of amino nitrogen (Craig *et al.* 1953). However, Ingram (1953) failed to demonstrate the presence of an N-terminal amino acid in bacitracin *A* by reductive methylation and Porath (1953), using the methods of Sanger & Thompson (1953) and Edman (1950), respectively, obtained neither a dinitrophenyl (DNP) derivative nor a phenylthiohydantoin corresponding to an N-terminal amino acid. Newton & Abraham (1953*a*) and Lockhart, Newton & Abraham (1954) reported that the DNP derivative of bacitracin *A* appeared to give DNP-isoleucine on hydrolysis, but that the yield was much less than that expected from one N-terminal isoleucine residue per mole. The nature of the third basic group in bacitracin *A* therefore remained uncertain.

Bacitracin *A* was also found to contain a latent thiol group. This group, forming part of a cysteine residue, was rapidly unmasked when the antibiotic was treated with N hydrochloric acid at 100°, and its liberation was accompanied by the disappearance of an absorption band at 254 mμ. (Newton & Abraham, 1953*a*; Craig, Weisiger, Hausmann & Harfenist, 1952). Newton & Abraham (1953*b*) discussed the possibility that the sulphur atom formed part of a thiazoline ring.

These facts suggested that bacitracin *A* was a polypeptide with unusual structural features. Further investigation of its structure appeared to fall into two related parts: the determination of the amino acid sequence in the polypeptide chain, and the determination of the way in which this chain is utilized to form the specific ring systems present in the molecule.

Lockhart *et al.* (1954) reported that the sequence



occurred in bacitracin *A*. The present paper describes further work on the amino acid sequence in bacitracin *A*. Some of the work, however, was carried out with material which contained bacitracin *B* as well as bacitracin *A*. In addition to the amino acids obtained from bacitracin *A*, hydrolysis of bacitracin *B* gives an amino acid which behaves like valine on paper chromatograms (Craig, Weisiger, Hausmann & Harfenist, 1952; Newton & Abraham, 1953*a*). An indication was obtained of the position of the 'valine' residue in the amino acid sequence of bacitracin *B*. The structure of this amino acid is uncertain, since it was isolated by Craig, Weisiger, Hausmann & Harfenist (1952) and reported to be different from valine.

METHODS

The methods used in the present work were similar to those employed by Sanger & Tuppy (1951) and by Sanger & Thompson (1953) for determining amino acid sequences. Peptides formed on partial hydrolysis were separated by ionophoresis in a four-compartment cell (Synge, 1951) and then by chromatography and ionophoresis on paper. The amino acid residues in the peptides were determined by complete hydrolysis and the N-terminal residues by reaction with 1:2:4-fluorodinitrobenzene (FDNB).

Antibacterial activity

Antibacterial activities were expressed in International Units (Humphrey, Lightbown, Mussett & Perry, 1953). The method of assay was similar to that of Brownlee *et al.* (1948). *Corynebacterium xerosis* was used as the test organism (Newton & Abraham, 1953*a*).

Bacitracin used for hydrolysis

Two samples of bacitracin (lot 311 and lot 25) were kindly supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A. Lot 311 (45 i.u./mg.) appeared to consist of bacitracins *A* and *B* together with smaller amounts of bacitracins *C*, *D*, *E* and *F* (Newton & Abraham, 1953*a*). Lot 25 (61 i.u./mg.) probably consisted mainly of bacitracin *A*, together with some bacitracin *B*, and possibly *D*. Its ultraviolet absorption spectrum in the region 230–

320 mμ. ($\lambda_{max.}$ 254 mμ.; $E^{1\%}_{1\,cm.}$ 25) was similar to that of bacitracin *A*. Lot 311 was used for preparing bacitracin *A*. Experiments were also carried out on lot 311 and lot 25 without further purification.

In some cases the latent thiol group in bacitracin was oxidized to —SO_3H before hydrolysis. Lot 311 was also inactivated by acid and the product converted into an *S*-methyl derivative, and lot 25 was inactivated by acid and then desulphurized by treatment with Raney nickel. It was intended in this way to obviate difficulties which might otherwise arise from the uncontrolled oxidation of the thiol group during the experiments. It was also thought that changes in the potential cysteine residue might facilitate the identification of some of the peptides formed on hydrolysis, or that they might be accompanied by other changes which would help to throw light on the structure of the molecule.

Bacitracin A. Bacitracin *A* was prepared by counter-current distribution of lot 311 (6 g.) in a solvent system consisting of *iso*amyl alcohol–*n*-butanol (4:1, by vol.) and 0·005 M potassium phosphate buffer pH 6·8 (Newton & Abraham, 1950). The distribution was carried out in eleven separating funnels (numbered 0–10), using 150 ml. of each phase of the system. The lower (aqueous) phase was mobile. After the 7th fundamental transfer, no new lower layer was added to the first funnel, and the upper layer was discarded, since it contained less than 2% of bacitracin *A* (Newton & Abraham, 1950). Similar discards were made after the 9th, 11th, 13th, 17th, 24th and 26th transfers to reduce the labour involved in making transfers by hand. On the 11th transfer the first member of the withdrawn series was removed. The distribution was stopped when nineteen withdrawn fractions had been collected and only three funnels of the fundamental series remained.

Photometric ninhydrin estimations of the material in the various fractions and estimations of the distribution of antibacterial activity were made by the methods of Newton & Abraham (1950). The optical densities of the fractions of the withdrawn series (diluted 1:3 with ethanol) were measured at 254 mμ. in a Beckman model DU spectrophotometer.

The ratio of antibacterial activity to ninhydrin colour density, or to the optical density at 254 mμ., was highest, and nearly constant, for the fractions withdrawn during transfers 23 to 30. The partition coefficient of the material in these fractions, which was estimated from the optical densities at 254 mμ., using the formula of Newton & Abraham (1952), was 1·07.

The fractions withdrawn during transfers 23–30 were pooled and concentrated in the manner described by Newton & Abraham (1950). The concentrate (90 ml., pH 4·0) was then freeze-dried, yielding 700 mg. of product with an activity of 72 i.u./mg.

This preparation of bacitracin *A* showed an absorption maximum at 254 mμ. ($E^{1\%}_{1\,cm.}$ = 21·4). After hydrolysis with 6N-HCl at 105°, a paper chromatogram run with butanol–acetic acid (Woiwod, 1949) showed the typical amino acid pattern of bacitracin *A*. Only a trace of 'valine' was detected, indicating that very little bacitracin *B* was present.

S-Methyl derivative of acid-inactivated bacitracin. Bacitracin (500 mg., lot 311) was dissolved in 0·5 N-HCl (10 ml.) and the solution heated at 100° for 20 min. The solution was freeze-dried and the residue dissolved in liquid NH_3 (about 30 ml.). Sodium was added in small pieces until the solution developed a permanent blue colour. Methyl iodide (0·1 ml.) was then added and the NH_3 allowed to evaporate. The residue was dissolved in water (25 ml.) and inorganic salts were removed by ionophoresis in the four-compartment cell. The solution from the specimen compartment (pH 6·5) was freeze-dried, yielding a white powder (125 mg.). After complete hydrolysis of this material with 6N-HCl at 105° for 24 hr., a paper chromatogram run with butanol–acetic acid showed that the spot due to cystine was absent, and a new spot due to *S*-methyl cysteine (R_F = 0·39) had appeared.

Desulphurized acid-inactivated bacitracin. Bacitracin (493 mg., lot 25) was inactivated by acid as in the preceding experiment, and then treated with Raney nickel as described by Newton & Abraham (1953*b*). The product (400 mg.) was contaminated with inorganic material. It was placed in the specimen compartment of a four-compartment cell (Synge, 1951) and subjected to ionophoresis (the pH being maintained at 6·5) until the current fell to 33 mA at 500 V. The solution was centrifuged to remove a small precipitate of alumina and then freeze-dried. The residue weighed 216 mg. After complete hydrolysis of this material with 6N-HCl at 105° for 24 hr., a paper chromatogram run with butanol–acetic acid (Woiwod, 1949) showed the typical amino acid pattern of bacitracin, except that the spot due to cystine was greatly diminished and a new spot due to alanine had appeared.

Oxidation of the sulphur in bacitracin to —SO_3H. This was carried out with performic acid, using the method of Sanger (1949).

Partial hydrolysis

Samples of bacitracin were hydrolysed in 11 N-HCl in sealed tubes at 37° (Sanger & Tuppy, 1951) for times varying from 1 to 10 days.

The photometric ninhydrin reaction (Moore & Stein, 1948) was used to make an approximate estimate of the average chain length of the resulting peptides. The average chain length was calculated from the amount of colour developed, a correction being made to allow for the fact that one mole of NH_3 was probably liberated at an early stage of the hydrolysis. Bacitracin *A* behaves as though it contained a labile amide group in addition to eleven or twelve amino acid residues. When preparations of desulphurized, acid-inactivated bacitracin or the *S*-methyl derivative of acid-inactivated bacitracin were used, which no longer contained 'amide' nitrogen, no correction was necessary.

Preliminary experiments showed that the average chain length fell rapidly to 4 in 24 hr. and then slowly decreased to 2·5 in 6 days and 2·0 in 10 days.

Separation of the peptides

Ionophoresis in a four-compartment cell. A preliminary fractionation of the peptides formed on partial hydrolysis of about 100 mg. of material was usually carried out in a four-compartment cell similar to that described by Synge (1951). Strongly basic peptides migrated into the cathode compartment, neutral peptides remained in the specimen compartment, and strongly acidic peptides migrated into the acetic acid compartment.

One-dimensional paper chromatograms. The solutions from the four-compartment cell were evaporated separately to dryness *in vacuo* and each residue dissolved in about 0·1 ml. water. Chromatograms of the resulting solutions were run with butanol–acetic acid (Woiwod, 1949) on separate sheets of Whatman no. 1 paper. Twenty spots of a solution (each 5·6 μl.) were placed on a sheet of paper at 12·5 mm. intervals, and on either side of them two spots were placed 25 mm. further away. After running the

chromatogram, vertical strips which contained the material from the outer spots were cut off each side of the paper. These strips were sprayed with ninhydrin solution and heated at 105° for 10 min. The resulting pattern served as a guide, which enabled the major portion of the chromatogram to be cut into horizontal strips carrying peptides and amino acids which had been separated from each other. These substances were eluted from the strips with water, using an apparatus similar to that of Kemble & Macpherson (1954) except that the trough was of stainless steel. The eluates were allowed to drop into Pyrex tubes (1·0 × 7·5 cm.) and evaporated to dryness *in vacuo*. In some cases, in which the peptides were not sharply resolved, it was found that a clearer separation could be obtained by subjecting the separate eluates to paper chromatography, using butanol–acetic acid.

Ionophoresis on paper. In most cases, the homogeneity of the peptides obtained from paper chromatograms was tested by ionophoresis at pH 7·0 on sheets of Whatman no. 1 paper 36 cm. long, in an apparatus similar to that of Flynn & Mayo (1951). The buffer was prepared by adding 36·3 ml. of commercial collidine (distilled under N_2; b.p. 54°/12 mm.) and 185 ml. of N acetic acid to 3·86 l. of water (Newton & Abraham, 1954). If a fraction was shown to consist of more than one component by ionophoresis, 10 spots (each 7·2 µl.) were placed at 12·5 mm. intervals on a sheet of paper together with a further spot at each end to act as a guide. After 500 v had been applied to the ends of the paper for 2·5 hr., the guide strips were developed with ninhydrin and the position of the resulting spots enabled the separate components from the main series of spots to be eluted in the manner used with paper chromatograms.

Other procedures. In a few cases the methods used were different from those described above. They included two-dimensional paper chromatography and ionophoresis on paper at pH 3.

Two-dimensional paper chromatograms were run first with butanol–acetic acid (Woiwod, 1949) and then with a collidine–lutidine mixture (Dent, 1948), using about 1 mg. of peptide (in 6·3 µl.) on each of four sheets of Whatman no. 3 MM paper. One of the sheets was sprayed with ninhydrin and the pattern obtained was used to estimate the position of the peptides on the untreated sheets. The areas of paper round these positions were cut out and the peptides eluted with water.

Ionophoresis on paper at pH 3 was carried out in 0·05 N acetic acid for 5 hr., using a potential gradient of 14 v/cm. This procedure was used with peptide mixtures in which the sulphur had been oxidized to $—SO_3H$, in the hope that highly acidic peptides containing cysteic acid would be readily separated from the remainder (Sanger & Thompson, 1953).

Analysis of peptides

Peptides formed on partial hydrolysis which appeared to have been obtained in a homogeneous form were hydrolysed in 6 N-HCl at 110° for 20 hr. The resulting amino acids were determined by chromatography and ionophoresis on paper. Most of the amino acids present in bacitracin could easily be distinguished on chromatograms run with butanol–acetic acid (Woiwod, 1949). Lysine was distinguished from ornithine by ionophoresis on paper for 2·5 hr. in the collidine acetate buffer (pH 7·0) previously described, using a potential gradient of 14 v/cm. on sheets of Whatman no. 1 paper 36 cm. long. Ornithine migrated slightly faster towards the cathode than lysine, and provided that the amount of each of these amino acids was less than 4 µg. and the volume of the solution applied to the paper did not exceed 2 µl., two distinct spots were formed when both were present. Histidine migrated about one-third as far as lysine. Leucine was well separated from isoleucine on paper chromatograms by using the *tert.*-amyl alcohol–water system of Work (1949). However, there is no evidence that isoleucine would have been separated from *allo*isoleucine.

In early experiments, N-terminal amino acids in the peptides were determined by the method of Sanger & Thompson (1953). Later this method was modified in an attempt to reduce the formation of 2:4-dinitrophenol, by using trimethylamine carbonate instead of trimethylamine. The peptide (50–150 µg.) was dissolved in 0·1 ml. of a 1·5% (w/v) solution of trimethylamine carbonate (pH 9·3), FDNB (0·01 ml.) in ethanol (0·2 ml.) was added, and the reaction allowed to proceed in the dark for 2·5 hr. The ethanol was removed *in vacuo*, 0·24 ml. of 1% (w/v) trimethylamine carbonate was added and excess FDNB extracted from the resulting solution with ether. The aqueous solution was evaporated to dryness *in vacuo*, the residue dissolved in 0·1 ml. of 6 N-HCl and the solution heated in a Pyrex tube under N_2 for 9 hr. at 105°. The hydrolysate was diluted with 2 vol. of water and the resulting solution shaken 3 times with an equal vol. of diethyl ether to extract the ether-soluble DNP-amino acids. Di-DNP-histidine (if present) was extracted with *n*-butanol. The extracted DNP-amino acids were run on paper chromatograms, using the system of Blackburn & Lowther (1951). The amino acids and any water-soluble DNP derivatives were studied on paper chromatograms run with butanol–acetic acid and by ionophoresis on paper at pH 7·0.

RESULTS

The results obtained from the various experiments are shown in Tables 1–4 and Fig. 1. In Fig. 1 the results obtained when mixtures of peptides were

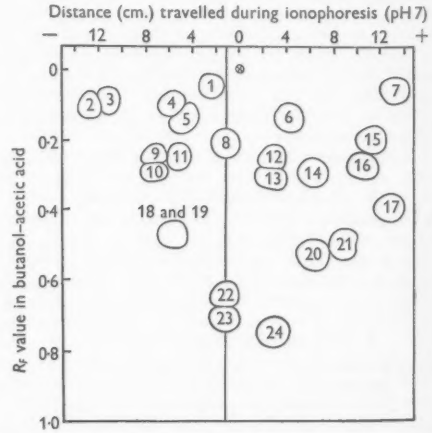


Fig. 1. Resolution of peptides from bacitracin *A* by chromatography and ionophoresis on paper (see Table 1). The central vertical line indicates the position of substances that have no net charge at pH 7.

run on paper chromatograms, eluted, and then subjected to ionophoresis on paper, have been brought together in a single diagram. The abbreviations used for amino acids are those recommended by Brand & Edsall (1947) and the methods of writing peptide structures are those of Sanger & Tuppy (1951). When leucine has not been distinguished from isoleucine the symbol 'Leu' is used.

Column 2 shows the R_F values on paper chromatograms run with butanol–acetic acid. Column 3 shows the distance which the spots moved during ionophoresis on paper. Unless otherwise stated, this was carried out at pH 7·0 for 2·5 hr. with a potential of 500 v in the manner previously described. Distances from the origin towards the cathode are given a negative sign. Substances with no net charge at pH 7·0 were carried about 1 cm. towards the cathode by endosmosis. Column 4 of the tables shows the amino acids detected on complete hydrolysis and the X's in column 5 indicate their approximate strength. Column 6 shows the relative strength of the amino acids remaining in the aqueous solution after the extraction of the hydrolysed DNP-peptide solution with diethyl ether and, in the case of peptides containing histidine, *n*-butanol. Column 7 shows the DNP-amino acid identified in the extract. In column 9, which contains cross references to apparently identical peptides, the first figure indicates the number of the table in which the peptide occurs, and subsequent figures the spot number.

A number of the peptides formed on partial hydrolysis of bacitracin appeared to yield cysteic acid on complete hydrolysis even though they came from material which had not been oxidized with performic acid. Some oxidation of the —SH group to an $—SO_3H$ group occurred in the four-compartment cell, in which the solutions were stirred by a stream of air. The reason why the oxidation proceeded beyond the —S—S— stage is not clear; this was never observed with the mixture of amino acids formed by complete hydrolysis of bacitracin.

Experiment 1. *Peptides from bacitracin* A

Bacitracin *A* was hydrolysed with 11 N-HCl for 6 days at 37°, the HCl was removed *in vacuo*, and the partial hydrolysate was oxidized with performic acid. The product was separated into acidic, neutral, and basic fractions in the four-compartment cell and the peptides in these fractions were further resolved by paper chromatography and subsequent ionophoresis on paper at pH 7·0 (Fig. 1). In other experiments the oxidation with performic acid was omitted, or was carried out before hydrolysis.

The peptides are listed in Table 1. Peptides 1–18, 20–22, and 24 were obtained from bacitracin *A* oxidized after partial hydrolysis, peptide 25 was obtained from bacitracin *A* oxidized before hydrolysis, and peptides 19 and 23 were obtained from unoxidized material. Peptides 1–5, 8–11 and 18–19 were obtained from the cathode compartment of the four-compartment cell, peptides 6, 12 and 23 from the specimen compartment, and peptides 7, 13–17, 20–22, and 24 and 25 from the acetic acid compartment.

Experiment 2. *Peptides from commercial bacitracin*

Peptides obtained from a sample of commercial bacitracin (lot 311, 45 i.u./mg.) and from the same material oxidized with performic acid are listed in Table 2. Partial hydrolysis was effected in both cases with 11 N-HCl for 6 days at 37°.

Peptides 1–13 and 18 were obtained from the unoxidized sample of bacitracin. Peptides 7, 8, 9, 11 and 13 were obtained from paper chromatograms of the acidic, neutral, and basic fractions from the four-compartment cell. Peptides 1–6 were obtained by the same procedure followed by ionophoresis on paper at pH 7·0. This readily separated 1 from 2. Peptides 10 and 12 were obtained by oxidizing the neutral fraction from the four-compartment cell with performic acid and subjecting the product to further ionophoresis in the four-compartment cell. Peptide 18 was obtained by subjecting a sample of the hydrolysate to ionophoresis on paper at pH 7·0.

Peptides 14–17 and 19–21 were obtained from the oxidized sample of bacitracin. Part of the hydrolysate was subjected to ionophoresis in the four-compartment cell and the resulting acidic fraction yielded peptides 14–17 on a paper chromatogram. The remainder of the hydrolysate was subjected to ionophoresis on paper in 0·05 N acetic acid, when all the peptides migrated towards the cathode except some containing cysteic acid. Spots which behaved as neutral or acidic were eluted and yielded peptides 19–21 on a paper chromatogram.

Experiment 3. *Peptides from an* S*-methyl derivative of acid-inactivated bacitracin*

Peptides obtained from the *S*-methyl derivative of acid-inactivated bacitracin (lot 311, 45 i.u./mg.) after hydrolysis with 11 N-HCl at 37° for 10 days are listed in Table 3. The cysteine residue in bacitracin had been converted into *S*-methyl cysteine, which was easily identified on a paper chromatogram run with butanol–acetic acid.

The hydrolysate was separated into neutral, basic, and acidic fractions in the four-compartment cell and the peptides in these fractions were further resolved by paper chromatography and by ionophoresis on paper at pH 7·0. Peptides 1–10 came from the neutral fraction, 11 from the basic fraction, and 12 from the acidic fraction.

Experiment 4. *Peptides from desulphurized acid-inactivated bacitracin*

Peptides obtained from desulphurized acid-inactivated bacitracin (lot 25; 61 i.u./mg.), after hydrolysis with 11 N-HCl for 8·5 days, are listed in Table 4. The hydrolysate was separated into acidic, neutral, and basic fractions in the four-compartment cell and the peptides in these fractions were resolved by paper chromatography and subsequent ionophoresis on paper at pH 7·0. Peptides 1–7 were obtained from the specimen compartment of the four-compartment cell, 8 from the acetic acid compartment and 9–14 from the cathode compartment.

Table 1. *Peptides from bacitracin* A

Spot no. (Fig. 1)	R_F value (butanol–acetic acid)	Migration (pH 7·0) in cm.	Amino acids present	Strength of amino acid after Hydrolysis	Strength of amino acid after DNP treatment	DNP-amino acid identified	Structure*	Identical with†
1	0·055	−2·2	His	× ×	−	His	His.[Asp, Asp, Lys, Orn]	.
			Asp	× × ×	× ×			
			Lys	× ×	× ×			
			Orn	× ×	−			
			'Leu'	?	−			
2	0·11	−12·0	Orn	× × ×	.	.	Ornithine	2·2 & 3·11
3	0·095	−11·0	Lys	×	.	.	[Lys, Orn]	.
			Orn	×	.			
			'Leu'	?	.			
4	0·10	−5·3	Asp	× ×	−	.	Asp.[Lys, Orn, 'Leu']	2·1 & 3·1
			Lys	× ×	× ×			
			Orn	× ×	×‡			
			'Leu'	×	−			
5	0·13	−4·5	His	× × ×	.	.	Histidine	2·8
6	0·14	5·0	His	× ×	−	.	His.Asp, or His.Asp, Asp	3·3
			Asp	× × ×	× ×			
7	0·07	13·5	$CySO_3H$	× × ×	.	.	Cysteic acid	2·21
8	0·21	−1·0	Asp	× × ×	.	.	[Asp, Asp, Lys, Orn, 'Leu']	.
			Lys	× ×	.			
			Orn	× ×	.			
			'Leu'	×	.			
9	0·26	−7·0	Lys	× ×	−	.	Lys.[Orn, 'Leu']	.
			Orn	× ×	×‡			
			'Leu'	× ×	×			
10	0·27	−7·0	Orn	× × ×	×‡	Orn	Orn.'Leu'	.
			'Leu'	× × ×	× ×			
			His	×	−			
			Phe	?	−			
11	0·28	−5·0	Phe	× ×	.	.	[Phe, His]	3·4 & 4·4
			His	× × ×	.			
			Lys	×	.			
			Orn	×	.			
			'Leu'	×	.			
12	0·26	3·0	Asp	× ×	×	Asp &/or Glu §	[Asp, Asp, Glu, Lys, Orn, 'Leu']	.
			Glu	×	×			
			Lys	×	×			
			Orn	×	×‡			
			'Leu'	×	×			

			'Orn'	×	×			
			'Leu'	×	×			
13	0·29	3·0	Asp	× × ×	.	.	[Asp, Glu, Lys, Ileu]	.
			Glu	× ×	.			
			Lys	× ×	.			
			Ileu	× ×	.			
14	0·29	6·5	$CySO_3H$	× ×	.	.	[$CySO_3H$, 'Leu', Glu, Lys, Asp]	.
			'Leu'	× ×	.			
			Glu	× ×	.			
			Lys	× ×	.			
			Asp	× ×	.			
15	0·21	11·0	Asp	× × × ×	.	.	Aspartic acid	2·11
16	0·28	10·0	Glu	× × ×	.	.	Glutamic acid	2·14
17	0·41	13·0	$CySO_3H$	× × ×	×	$CySO_3H$	$CySO_3H$.[Leu, Glu]	2·19
			Leu	× × ×	× × ×			
			Glu	× × ×	× × ×			
18	0·48	−5·5	Orn	× × × ×	×‡	Orn	Orn.[Ileu, P]	.
			Ileu	× × × ×	× × ×			
			Unknown (P)‖	× × ×	× ×			
19	0·48	−5·5	Orn	× × × ×	−	Orn	Orn.[Ileu, Phe]	2·6, 3·7 & 4·14
			Ileu	× × × ×	× ×			
			Phe	× × × ×	× ×			
20	0·55	6·0	Leu	× × × ×	×	Leu	Leu.Glu	2·13, 3·12 & 4·8
			Glu	× × × ×	× × ×			
21	0·50	9·0	$CySO_3H$	× × × ×	×	$CySO_3H$	$CySO_3H$.Leu	2·16 & 2·20
			Leu	× × × ×	× × × ×			
22	0·63	−1·0	Ileu	× × × ×	.	.	Isoleucine¶	3·8
23**	0·68	−1·0	Phe	× × × ×	×	?	Phe.'Leu'	.
			'Leu'	× × × ×	× × × ×			
24	0·75	3·3	Asp	× ×	×	Asp &/or Glu	Glu.[Asp, Lys, 'Leu']††	.
			Glu	× ×	−			
			Lys	× ×	× ×			
			'Leu'	× ×	×			
25	0·58	.	'Leu'	× × × ×	× × ×	'Leu'	'Leu'.[$CySO_3H$, 'Leu', Glu]	2·17
			$CySO_3H$	× × ×	× × ×			
			Glu	× × ×	× × ×			

* Square brackets indicate that the sequence of amino acids inside the brackets has not been determined.

† The first figure indicates the number of the table in which the peptide occurs and the subsequent figures the spot no.

‡ Identified as δ-DNP-ornithine.

§ The DNP-amino acid was accompanied by material which remained at the origin on paper chromatograms (Blackburn & Lowther, 1951) and showed a pale green fluorescence in ultraviolet light (365 mμ.).

‖ On paper chromatograms run with butanol–acetic acid and developed with ninhydrin this substance appeared as a grey-blue spot (R_F 0·69) below leucine. The colour was similar to that given by phenylalanine.

¶ This isoleucine came from the acidic fraction from the four-compartment cell. It may have been formed by subsequent hydrolysis of an acidic peptide.

** This peptide was resolved on a two-dimensional chromatogram using butanol–acetic acid and collidine–lutidine (Dent, 1948). R_F 0·33 in collidine–lutidine.

†† The relationship of this peptide to that forming spot 13 and the reason for its high R_F value are uncertain.

Table 2. *Peptides from commercial bacitracin*

Spot no.	R_F value (butanol–acetic acid)	Migration (pH 7·0) in cm.	Amino acids present	Strength of amino acid after Hydrolysis	Strength of amino acid after DNP treatment	DNP-amino acid identified	Structure	Identical with
1	0·10	−5·3	Asp	× ×	–	Asp	Asp.[Lys, Orn, 'Leu']	1·4 & 3·1
			Lys	× ×	× ×			
			Orn	× ×	×*			
			'Leu'	× ×	×			
2	0·11	−12·0	Orn	× × × ×	.	.	Ornithine	1·2 & 3·11
3	0·36	−7·0	Lys	× ×	.	.	[Lys, Orn, 'Leu']	.
			Orn	× ×	.			
			'Leu'	× ×	.			
4	0·40	−7·0	Orn	× ×	.	.	[Orn, 'Leu', Phe, His]	.
			'Leu'	× ×	.			
			Phe	× ×	.			
			His	× ×	.			
5	0·42	−5·5	Orn	× × ×	.	.	[Orn, Val, Phe]	.
			Val	× × ×	.			
			Phe	× × ×	.			
6	0·48	−5·5	Orn	× × × ×	–	Orn	Orn.['Leu', Phe]	1·19, 3·7 & 4·14
			'Leu'	× × × ×	× ×			
			Phe	× × × ×	× ×			
7	0·64	.	None†	.	.	.	.	.
8	0·13	−4·5	His	× × × ×	.	.	Histidine	1·5
9	0·63	.	Phe	× × ×	.	.	[Phe, 'Leu', $CySO_3H$]‡	.
			'Leu'	× × × ×	.			
			$CySO_3H$	× × ×	.			
10	0·65	−1·0	Phe	× × ×	.	.	[Phe, 'Leu']	.
			'Leu'	× × ×	.			
11	0·21	11·0	Asp	× × × ×	×	Asp	Aspartic acid	1·15
12	0·34	.	$CySO_3H$	× × ×	.	.	[$CySO_3H$, 'Leu']	.
			'Leu'	× × × ×	.			

13	0·55	6·0	Leu Glu	× × × × × × × ×	× × × × ×	Leu	Leu.Glu	1·20, 3·12 & 4·8
14	0·28	10·0	Glu	× × × ×	.	.	Glutamic acid	1·16
15	0·36	.	'Leu' $CySO_3H$	× × × × × × × ×	× × × ×	'Leu'	'Leu'.$CySO_3H$	.
16	0·50	9·0	$CySO_3H$ 'Leu'	× × × × × ×	× × × ×	$CySO_3H$	$CySO_3H$.'Leu'	1·21 & 2·20
17	0·58	.	$CySO_3H$ Glu 'Leu'	× × × × × × × × × ×	× × × × × × × × ×	'Leu'	'Leu'.[$CySO_3H$, 'Leu', Glu]	1·25
18	0·28	−3·6	His Asp Lys Orn 'Leu'	× × × × × × × × × ×	. . . . .	.	[His, Asp, Lys, Orn, 'Leu']	.
19§	0·41	‖	$CySO_3H$ 'Leu' Glu	× × × × × × × × ×	– × × × ×	$CySO_3H$	$CySO_3H$.[Leu, Glu]	1·17
20	0·50	‖	$CySO_3H$ Leu	× × × × × × × ×	– × × ×	$CySO_3H$	$CySO_3H$.Leu	1·21 & 2·16
21	0·07	‖	$CySO_3H$	× ×	.	.	Cysteic acid	1·7

* Identified as δ-DNP-ornithine.

† This spot gave no colour with ninhydrin but showed a pale blue fluorescence in ultraviolet light (365 mμ.). It yielded no amino acids on hydrolysis with 6N-HCl at 110°.

‡ This peptide appeared to yield cysteic acid on hydrolysis, although it came from the specimen compartment of the four-compartment cell. Oxidation of the sulphur may have occurred in subsequent manipulations.

§ This spot gave initially with ninhydrin a yellow colour, which later changed to purple.

‖ These peptides were subjected to ionophoresis at pH 3 on Whatman no. 3 MM paper (36 cm. long) at 500 v for 5 hr. 19 and 20 migrated 1 cm. towards the cathode and 21 migrated 11·5 cm. towards the anode.

Table 3. *Peptides from an* S*-methyl derivative of acid-inactivated bacitracin*

Spot no.	R_F value (butanol–acetic acid)	Migration (pH 7·0) in cm.	Amino acids present	Strength of amino acid after: Hydrolysis	Strength of amino acid after: DNP treatment	DNP-amino acid identified	Structure	Identical with
1	0·10	−5·3	Asp	× ×	×	Asp	Asp.[Lys, Orn, Ileu]	1·4 & 2·1
			Lys	× ×	× ×			
			Orn	× ×	×*			
			Ileu	×	×			
2	0·09	−1·0	Asp	× ×	×	Asp & Glu	Asp.(Glu).[Lys, Orn, Ileu.]	4·3
			Glu	× ×	−			
			Lys	× ×	× ×			
			Orn	× ×	×*			
			Ileu	× ×	×			
3	0·14	5·0	His	× ×	−	His	His.Asp, or His.Asp.Asp	1·6
			Asp	× ×	× ×			
4	0·28	−5·0	Phe	× ×	·	·	[Phe, His]	1·11 & 4·4
			His	× ×	·			
5	0·37	−7·5	Lys	× ×	−	·	[Lys, Orn, Ileu]	·
			Orn	× ×	−			
			Ileu	× ×	× ×			
6†	0·38	−2·0	Asp	× ×	−	Asp‡	Asp.(Ileu).Lys	·
			Lys	× ×	× ×			
			Ileu	× ×	−			
7	0·48	−5·5	Orn	× × ×	?*	Orn	Orn.[Ileu, Phe]	1·19, 2·6 & 4·14
			Ileu	× × ×	× ×			
			Phe	× × ×	× ×			
8	0·63	−1·0	Ileu	× × ×	·	·	Isoleucine	1·22
9	0·72	−1·0	Ileu	× × × ×	× × ×	Ileu	Ileu.[Phe, Ileu, $CySCH_3$]	·
			Phe	× × ×	× × ×			
			$CySCH_3$	× × ×	× × ×			
10	0·79	−1·0	Ileu	× × ×	×	Ileu	Ileu.[$CySCH_3$, Leu]	·
			$CySCH_3$	× × ×	× × ×			
			Leu	× × ×	× × ×			
11	0·11	−12·0	Orn	× × ×	·	·	Ornithine	1·2 & 2·2
12	0·55	6·0	Leu	× × ×	×	Leu	Leu.Glu	1·20, 2·13 & 4·8
			Glu	× × ×	× × ×			

* Identified as δ-DNP-ornithine.
† This spot gave a yellow-brown colour with ninhydrin.
‡ The DNP-aspartic acid was accompanied by material which remained at the origin on a paper chromatogram (Blackburn & Lowther, 1951) and showed a bright green fluorescence in ultraviolet light (365 mμ.). A trace of material which behaved like DNP-isoleucine was also present.

DISCUSSION

In so far as their composition could be ascertained by the use of paper chromatography, almost all the peptides obtained from bacitracin *A* and from the two commercial samples of bacitracin were consistent with the same amino acid sequence, even though the commercial samples contained some bacitracin *B* and other bacitracins. The different bacitracins show strong similarities in the amino acid patterns given by their hydrolysates on paper chromatograms (Newton & Abraham, 1953*a*) and it may be that they also resemble each other in the order of amino acids in the peptide chains.

Certain peptides were frequently encountered in relatively large amounts in different experiments, particularly, leucylglutamic acid, β-sulphoalanylleucylglutamic acid, β-sulphoalanylleucine and ornithyl[isoleucylphenylalanine]. Other peptides, especially those containing histidine and aspartic acid, or having glutamic acid as an N-terminal residue, were relatively rare. Free histidine and free aspartic acid appeared early during the hydrolysis. These facts indicated that there were preferential points of cleavage in the molecule.

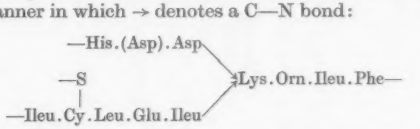
Since bacitracin *A* contains only one leucine residue, the peptides Leu.Glu (Table 1, spot 20) and $CySO_3H$.Leu (spot 21) indicate that peptide 17 is $CySO_3H$.Leu.Glu. Peptide 25 should therefore be Ileu.$CySO_3H$.Leu.Glu. The peptides Orn.-'Leu' (spot 10) and Orn.[Ileu, Phe] (spot 19) indicate that there is a sequence: Orn.Ileu.Phe. The peptides [Lys, Orn] (spot 3), Lys.[Orn, 'Leu'] (spot 9), Asp.[Lys, Orn, 'Leu'] (spot 4) and [Asp, Asp, Lys, Orn, 'Leu'] (spot 8) allow the latter sequence to be extended to Asp.Asp.Lys.Orn.-Ileu.Phe. The peptides His.Asp (spot 6) and His.[Asp, Asp, Lys, Orn] (spot 1) indicate that the histidine residue is linked to an aspartic acid residue. Hence, these peptides are consistent with the following sequences in bacitracin *A*:

(1) Ileu.CyS.Leu.Glu
(2) His.Asp.Asp.Lys.Orn.Ileu.Phe

In the latter sequence it remains uncertain whether one of the aspartic acid residues forms a branch from the main chain.

Many of the lower peptides obtained from partial hydrolysates of the samples of commercial bacitracin (Tables 2 to 4) would fit into these sequences. The peptide [His, Asp, Lys, Orn, 'Leu'] (Table 2, spot 18) suggests that one of the aspartic acid residues may form a branch in the chain. This will be indicated by the symbol (Asp).

The peptides [Asp, Glu, Lys, Ileu] (Table 1, spot 13) from bacitracin *A* and Asp.(Ileu).Lys (Table 3, spot 6) from commercial bacitracin indicated that the two sequences were linked together through an isoleucine residue in the following manner in which → denotes a C—N bond:



The peptides [Asp, Asp, Glu, Lys, Orn, 'Leu'] (Table 1, spot 12), [$CySO_3H$, 'Leu', Glu, Lys, Asp] (Table 1, spot 14) and Glu.[Asp, Lys, 'Leu'] (Table 1, spot 24) from bacitracin *A*, and the peptides Asp.(Glu).[Lys, Orn, Ileu] (Table 3, spot 2) and Asp.('Leu').[Asp, Lys, Orn], Asp.-('Leu').[Lys, Orn] and [Asp, Glu, Lys, Orn, 'Leu'] (Table 4, spots 1 to 3) from commercial bacitracin were consistent with such a linkage. This arrangement involves the assumption that there are three isoleucine residues, or potential isoleucine residues, in the molecule. Hence, two sets of peptides could be formed which contained a lysine, ornithine and one isoleucine residue.

There were indications of some peculiarity in the Asp.(Ileu).Lys fragment. After treatment with FDNB and hydrolysis, the peptides [Asp, Asp, Glu, Lys, Orn, 'Leu'] (Table 1, spot 12), Asp.-(Ileu).Lys (Table 3, spot 6) and Asp.('Leu').-[Lys, Orn, 'Leu'?] (Table 4, spot 9) yielded ether-soluble material which showed a green fluorescent spot at the origin on paper chromatograms when viewed under ultraviolet light (365 mμ.) and which did not correspond to the DNP derivative of a known amino acid. A similar fluorescent spot was obtained on chromatographing the ether-soluble material from a hydrolysate of DNP-bacitracin *A*. Although free glutamic acid, free aspartic acid, and free ornithine were found to be present in partial hydrolysates, free lysine was never observed, and none of the peptides which were allowed to react with FDNB yielded a mono-DNP-lysine on subsequent hydrolysis. These results showed that one or both of the amino groups of lysine were involved in particularly stable linkages.

The peptides [Phe, His] (Table 1, spot 11) from bacitracin *A* and Phe.His (Table 3, spot 4; Table 4, spot 4) and [Orn, 'Leu', Phe, His] (Table 2, spot 4) from commercial bacitracin indicated that histidine and phenylalanine residue should be linked together, in the sequence shown above, to form a ring. The resulting structure would then be identical with that put forward by Craig *et al.* (1954), but different from that suggested by Porath (1953).

One peptide appeared to be Phe.'Leu' (Table 1, spot 23). Others behaved as though they were [Phe, 'Leu', $CySO_3H$] (Table 2, spot 9), Ileu.[Phe, Ileu, $CySCH_3$] (Table 3, spot 9) and Ileu.[Phe, Ileu, Ala] (Table 4, spot 7), although the possibility that they were mixtures of dipeptides cannot be

† 1 of 2:4-aspartic acid was accompanied by material which remained at the origin on a paper chromatogram (Blackburn & Lowther, 1951) and showed a bright green fluorescence in ultraviolet light (365 mμ.). A trace of material which behaved like DNP-isoleucine was also present.

Table 4. *Peptides from desulphurized acid-inactivated bacitracin*

Spot no.	R_F value (butanol–acetic acid)	Migration (pH 7·0) in cm.	Amino acids present	Strength of amino acid after Hydrolysis	Strength of amino acid after DNP treatment	DNP-amino acid identified	Structure	Identical with
1	0·05	−1·0	Asp	× × ×	× ×	Asp & 'Leu'	Asp.('Leu').[Asp, Lys, Orn]	.
			Lys	× ×	× ×			
			Orn	× ×	×*			
			'Leu'	×	?			
2	0·05	−5·2	Asp	×	?	Asp & 'Leu'	Asp.('Leu').[Lys, Orn]	.
			Lys	× ×	× ×			
			Orn	× ×	×*			
			'Leu'	×	?			
3	0·09	−1·0	Asp	× × ×	.	.	[Asp, Glu, Lys, Orn, 'Leu']	3·2
			Glu	× × ×	.			
			Lys	× × ×	.			
			Orn	× × ×	.			
			'Leu'	× ×	.			
4	0·28	−5·0	Phe	× ×	–	Phe	Phe.His	1·11 & 3·4
			His	× ×	×†			
5	0·30	−4·0	Lys &/or Orn	× ×	.	.	[(Lys &/or Orn), 'Leu', Phe, His, Asp]	.
			'Leu'	× ×	.			
			Phe	× ×	.			
			His	× ×	.			
			Asp	× ×	.			
6	0·33	−7·0	Orn	× ×	.	.	[Orn, Lys, 'Leu']	.
			Lys	× ×	.			
			'Leu'	× ×	.			
7	0·71	−1·0	Ileu	× × × ×	× ×	Ileu	Ileu.[Phe, Ileu, Ala]	.
			Phe	× ×	× ×			
			Ala	× ×	×			
			Val	×	?			

8	0·55	6·0	Leu Glu	× × × × × × × ×	× × × ×	Leu	Leu.Glu	1·20, 2·13 & 3·12
9	0·16	−6·0	Asp Lys Orn 'Leu'	× × × × × × × ×	× × × ×* ×	Asp‡ & 'Leu'	Asp.('Leu').[Lys, Orn, 'Leu'?]	.
10	0·40	−1·0	Asp Lys Orn Ileu Phe	× × × × × × × × × × ×	. . . . .	.	[Asp, Asp, Lys, Orn, Ileu, Phe]	.
11	0·29	−7·5	Lys Orn Val	× × ×	. . .	.	[Lys, Orn, Val]	.
12	0·36	−7·5	Lys Orn 'Leu'	× × × × × × × × ×	– × ×* × ×	Lys?	Lys.[Orn, 'Leu']	.
13	0·36	−4·3	Asp Lys Orn 'Leu' Phe	× × × × × × × × × ×	? × ×* × ×	.	Asp.[Lys, Orn, 'Leu', Phe]	.
14	0·48	−5·5	Orn 'Leu' Phe Val	× × × × × × × × × ×	×* × × × × ?	Orn	Orn.['Leu', Phe]	1·19, 2·6 & 3·7
15	0·13	−2·3	Unknown§	× × ×	.	.	.	.

* Identified as δ-DNP-ornithine.

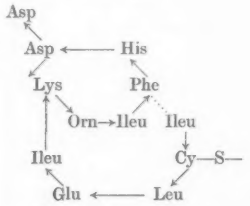
† Identified as gly-DNP-histidine.

‡ A trace of DNP-aspartic acid was accompanied by material which remained at the origin on a paper chromatogram (Blackburn & Lowther, 1951) and which showed a green fluorescence in ultraviolet light (365 mμ.).

§ This substance gave a positive Pauly reaction and a grey colour (which developed slowly) with ninhydrin. It migrated only half as far as histidine when subjected to ionophoresis on paper at pH 7. It appeared to be unchanged by treatment with 6N-HCl at 110°.

ignored. However, if the structure assigned to these peptides is correct they indicate that the phenylalanine residue should also be linked to the N-terminal isoleucine residue. It is evident that this arrangement could not be accommodated with an ordinary peptide bond. Further evidence about the structure of this part of the molecule might be obtained by identification of the unknown component of peptide 18 in Table 1 and of the substance responsible for spot 15 in Table 4.

The presence of an N-terminal isoleucine residue in bacitracin *A*, as suggested by Newton & Abraham (1953*a*), would account for the third basic group in the molecule and for the formation of DNP-isoleucine on hydrolysis of DNP-bacitracin *A*. Nevertheless, the low yield of DNP-isoleucine indicates that the isoleucine from which it is derived is abnormal. Possibly this is the residue which appears to give rise to *allo*isoleucine on complete hydrolysis of bacitracin *A*. In view of these facts the results of the present work appear to be best explained by assigning the following structure to bacitracin *A*, in which the dotted lines represent a tentative unknown linkage:



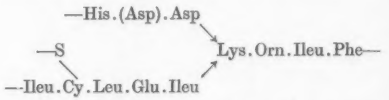
The glutamic acid or one of the aspartic acid residues in this structure could be present as glutamine or asparagine respectively. The latent thiol group could be accounted for if the sulphur atom, as suggested by Newton & Abraham (1953*a*) and by Craig *et al.* (1954), was incorporated in a thiazoline ring. The ring would involve the carboxyl group of the isoleucine residue that is adjacent to the cysteine residue and might contribute to the abnormal behaviour which this isoleucine appears to show.

The peptides [Orn, Val, Phe] (Table 2, spot 5) and [Lys, Orn, Val] (Table 4, spot 11), obtained from commercial bacitracin, presumably contain a so-called valine residue that is present in bacitracin *B*. They suggest that one way in which bacitracin *B* differs from bacitracin *A* is in containing the sequence Lys.Orn.Val.Phe instead of the sequence Lys.Orn.Ileu.Phe.

SUMMARY

1. Bacitracin *A* has been subjected to partial hydrolysis with acid and the resulting peptides fractionated by ionophoresis in a four-compartment cell, paper chromatography, and ionophoresis on paper. Similar experiments have been carried out with commercial bacitracin, and also with commercial bacitracin that has been inactivated with acid and subsequently converted into an *S*-methyl derivative or desulphurized.

2. Most of the peptides obtained from both bacitracin *A* and commercial bacitracin are consistent with the presence of the following sequence, in which a branch arises at the lysine residue:



3. Several peptides indicate that the histidine and phenylalanine residues in bacitracin *A* are linked together to form a ring. Further peptides suggest that the phenylalanine residue may also interact with the isoleucine residue that is next to the potential cysteine.

4. Peptides from commercial bacitracin which contain 'valine' suggest that the sequence Lys.Orn.Val.Phe. occurs in bacitracin *B*.

It is a pleasure to acknowledge help and advice from Dr G. G. F. Newton during the course of this work. We wish to thank Miss M. Read and Mrs A. Giles for technical assistance, and the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A., for generous gifts of bacitracin. One of us (I.M.L.) is indebted to the Medical Research Council for a Scholarship.

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Biochemical Studies of Toxic Agents

7. THE METABOLISM OF NAPHTHALENE IN ANIMALS OF DIFFERENT SPECIES

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(*Received* 5 *July* 1954)

Although from time to time various metabolites have been isolated from the urines of animals dosed with naphthalene, no systematic study of the influence of species on the metabolism of this hydrocarbon has hitherto been made. In the present work the metabolism of naphthalene in rabbits, rats, guinea pigs and mice has been studied. Paper chromatography has been used to examine the urines of the dosed animals and a number of metabolites have also been isolated. The metabolism of the hydrocarbon in guinea pigs and mice had not previously been investigated. Our findings show that although the optical activity of the metabolite 1:2-dihydronaphthalene-1:2-diol is influenced by species, most of the metabolic reactions which naphthalene has been shown to undergo occur in all four of the animal species studied.

CHROMATOGRAPHIC STUDIES

Reference compounds

1- *and* 2-*Naphthol.* These compounds were AnalaR reagents obtained from British Drug Houses Ltd., and each was recrystallized once from water before use.

1:2-*Dihydroxynaphthalene.* Finely powdered 1:2-naphthoquinone (1·0 g.) was stirred into water (15 ml.) and the suspension was warmed on a water bath with constant stirring. The mixture was then cooled to room temperature, centrifuged and the supernatant was discarded. The moist naphthoquinone was added in small amounts to 15 ml. of an aqueous solution of sodium dithionite (10%, w/v) and, when the additions had been completed, the mixture was left in the refrigerator overnight. The tan-coloured precipitate which formed was removed by filtration and dried *in vacuo*. The product was recrystallized twice from light petroleum (b.p. 60–80°) and yielded 0·341 g. of colourless needles, m.p. 104° (m.p.'s reported in this paper are uncorrected). Fieser & Fieser (1939) have reported that 1:2-dihydroxynaphthalene melts at 104°. (Found: C, 74·8; H, 5·1. Calc. for $C_{10}H_8O_2$: C, 75·0; H, 5·0%.)

1:4-*Dihydroxynaphthalene.* This was prepared from 1:4-naphthoquinone by the procedure just described. The compound melted at 177°. The m.p. given by Heilbron & Bunbury (1953) is 176°. (Found: C, 74·5; H, 4·8. Calc. for $C_{10}H_8O_2$: C, 75·0; H, 5·0%.)

1:2-*Dihydronaphthalene*-1:2-*diol.* Biosynthetic methods were used to obtain (±)-*trans*-1:2-dihydronaphthalene-1:2-diol. Naphthalene was administered to male hooded rats and 1:2-dihydronaphthalene-1:2-diol was isolated from the urine by the method described by Young (1947). The compound isolated from the urine of rats of the strain used in the present work showed no measurable optical rotation and it melted at 100°. (Found: C, 74·1; H, 6·1. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%.) The diacetate of the compound showed no optical rotation and melted at 74°. (Found: C, 68·7; H, 5·5. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.)

1-*Naphthyl glucosiduronic acid.* The method of Berenbom & Young (1951) was used to isolate this compound from the urine of rabbits dosed with 1-naphthol. The compound so obtained melted at 199°, $[\alpha]_D^{20}$ – 85° in ethanol (*c*, 1). The triacetyl methyl ester was prepared, m.p. 160°, $[\alpha]_D^{20}$ – 79° in $CHCl_3$ (*c*, 1). (Found: C, 59·5; H, 5·7; CH_3CO—, 27·7. Calc. for $C_{23}H_{24}O_{10}$: C, 60·0; H, 5·2; CH_3CO—, 28·0%.)

2-*Naphthyl glucosiduronic acid.* This was isolated in a similar way after rabbits were dosed with 2-naphthol. The compound melted at 153–154°, $[\alpha]_D^{20}$ – 96° in ethanol (*c*, 1). The triacetyl methyl ester had m.p. 127–128° and $[\alpha]_D^{0}$ – 35° in $CHCl_3$ (*c*, 1). (Found: C, 60·4; H, 5·3; CH_3CO—, 27·6. Calc. for $C_{23}H_{24}O_{10}$: C, 60·0; H, 5·2; CH_3CO—, 28·0%.)

Potassium 1-*naphthyl sulphate.* The method of Feigenbaum & Neuberg (1941) was used in order to synthesize this compound from 1-naphthol. The compound was converted into the *p*-toluidine salt by the procedure described by Barton & Young (1943). Treatment of the *p*-toluidine salt with KOH regenerated the pure potassium salt. (Found: total sulphate S, 12·4. Calc. for $C_{10}H_7O_4SK$, 12·2%.)

Potassium 2-naphthyl sulphate. This compound was prepared from 2-naphthol in a similar way to that just described. (Found: total sulphate S, 12·1. Calc. for $C_{10}H_7O_4SK$, 12·2%.)

Detection of compounds on paper chromatograms

1- and 2-Naphthol and 1:2- and 1:4-dihydroxynaphthalene were detected by spraying the papers lightly with a 0·15% (w/v) solution of diazotized sulphanilic acid in water followed by ammonia (sp.gr. 0·880). A second procedure for detecting these compounds was used, namely, spraying with a 0·1% (w/v) solution of 2-chloro-4-nitrobenzenediazonium naphthalene-2-sulphonate (NNCD reagent) in water (Heinrich & Schuler, 1947, 1948).

To detect 1:2-dihydronaphthalene-1:2-diol on paper chromatograms use was made of the finding by Young (1947) that mild acid treatment of this compound leads to the formation of naphthol. This change was effected on chromatograms by suspending the papers in fumes of HCl in a closed vessel for 10 min. at room temperature. The papers were then exposed to a current of air for a short period. They were then sprayed either with diazotized sulphanilic acid followed by ammonia, or with NNCD reagent. In this way colours characteristic of 1-naphthol were obtained.

1- and 2-Naphthylsulphuric acid (1- and 2-naphthyl hydrogen sulphate) and 1- and 2-naphthyl glucosiduronic acid were hydrolysed on chromatograms by suspending the papers in fumes of HCl at room temperature for 15 min. (sulphuric acid conjugates) or for 1 hr. (glucuronic acid conjugates). The naphthols liberated by acid hydrolysis were then detected as already described.

The colours observed on paper chromatograms when hydroxy derivatives of naphthalene are treated with the detecting reagents are shown in Table 1.

General methods

Dosing of animals. The animals were housed in metabolism cages designed to permit the collection of urine separate from faeces. Rabbits and guinea pigs were fed on a diet of cabbage and mixed bran and chaff. Rats and mice received a bread and milk diet with vitamin supplements. Each animal received a single dose of naphthalene (0·5 g./kg. body wt.) as a 20% (w/v) solution in arachis oil. Naphthalene was administered to rabbits by stomach tube. Rats were dosed by stomach tube and, in some experiments, by intraperitoneal injection. Guinea pigs and mice were dosed by intraperitoneal injection only. To facilitate the administration of naphthalene to rats and mice the animals were anaesthetized lightly with ether.

Preparation of urine extracts. Urine was collected for 2 days after the animals were dosed. The urine was filtered through glass wool, adjusted to pH 7, continuously extracted with peroxide-free ether for 24 hr. and the ether extract was taken to dryness. A brownish red residue was usually obtained, although that formed by evaporation of the ether extracts of guinea pig urine was sometimes reddish purple. The residue was dissolved in a small volume of ethanol and the solution was examined by paper chromatography for the presence of unconjugated hydroxy derivatives of naphthalene.

After extraction with ether, the urine was evaporated under reduced pressure to approximately one-tenth of its volume and the concentrate so obtained was shaken with an equal volume of *n*-butanol. The emulsion which formed was centrifuged and the butanol layer was examined by paper chromatography for the presence of conjugated hydroxy derivatives of naphthalene. It has been shown that the sulphuric acid and glucuronic acid conjugates of 1- and 2-naphthol are extracted from urine by *n*-butanol (Berenbom & Young, 1951).

Chromatographic procedures. Descending chromatography on Whatman no. 1 paper was used, and details of the solvent systems employed are included in Table 1. Each solvent mixture was shaken vigorously for 5 min. and left overnight to separate into two phases. The upper layer was used as the mobile phase and the lower layer as the stationary phase. The experiments were carried out in airtight glass tanks, and the chromatograms were developed after a period of equilibration of 16 hr. As conditions of constant temperature were not used in these experiments, chromatograms spotted with a mixture of reference compounds were always developed simultaneously with those used to examine metabolites of naphthalene.

Results

The identities of the metabolites listed in Table 1 were deduced from a comparison of their R_F values with those of reference compounds which were run simultaneously in the same solvent systems, and from the colours which the metabolites gave with diazo reagents, either before or after the chromatograms were treated with acid. When examined by paper chromatography, ether extracts and butanol extracts of the normal urines of each animal species were found to contain none of the compounds described in Table 1, including those for which no marker compounds were available.

After they had been dosed with naphthalene, rabbits, rats, guinea pigs and mice all excreted unconjugated 1- and 2-naphthol. Guinea pigs also excreted 1:2-dihydroxynaphthalene under these conditions, but this metabolite could not be detected in the urines of the other species studied. From the relative sizes and intensities of the spots obtained it appeared that the rabbits and rats excreted more 2-naphthol than 1-naphthol, that guinea pigs excreted 1- and 2-naphthol in approximately equal amounts and that mice excreted more 1-naphthol than 2-naphthol.

1:2-Dihydronaphthalene-1:2-diol and 1-naphthylsulphuric acid were formed from naphthalene in all four species and, in addition, rabbits, rats and mice excreted 1-naphthyl glucosiduronic acid. 2-Naphthylsulphuric acid and 2-naphthyl glucosiduronic acid were not detected in any of the urines studied, even those of rabbits and rats which contained larger amounts of 2-naphthol than 1-naphthol. As the R_F values of 1-naphthyl glucosiduronic acid and 1-naphthylsulphuric acid were close to those of the corresponding derivatives of 2-naphthol, it seemed possible that this may have accounted for the failure to detect these latter compounds. Experiments were therefore carried out in which quantities of 2-naphthyl glucosiduronic

Table 1. *The chromatographic examination of ether and butanol extracts of the urines of animals dosed with naphthalene*

R_F values, except those in black, which refer to papers treated with HCl fumes before spraying, refer to papers sprayed immediately. Colours of spots: DR, dark red; BR, brick red; O, orange; Y, yellow; P, purple. Solvent systems (volumes mixed): 1, *cyclo*hexane–water–acetic acid (5:4:1); 2, *cyclo*hexane–water–ammonia (sp.gr. 0·88) (5:4:1); 3, benzene–water–acetic acid (5:4:1); 4, benzene–water–ammonia (sp.gr. 0·88) (5:4:1); 5, *cyclo*hexane–benzene–water–ammonia (sp.gr. 0·88) (5:5:8:2); 6, 3 N-NH_4OH–3 N-$(NH_4)_2CO_3$–*n*-butanol (3:3:4); 7, 3 N-NH_4OH–3 N-$(NH_4)_2CO_3$–*iso*butanol (3:3:4).

Solvent system no.	R_F values				Colours		Metabolites
	Guinea pig	Mouse	Rat	Rabbit	Diazotized sulphanilic acid	NNCD reagent	
					Ether extracts		
1	0·80	—	—	—	P	BR	Unknown
	0·52	0·51	0·53	0·52	DR	Y	1-Naphthol
	0·36	0·37	0·37	0·36	O	Y	2-Naphthol
	0·01	—	—	—	P	P	1:2-Dihydroxynaphthalene
	0·01	**0·01**	**0·01**	**0·01**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
2	0·37	—	—	—	P	BR	Unknown
	0·24	0·24	0·24	0·23	DR	Y	1-Naphthol
	0·17	0·17	0·17	0·16	O	Y	2-Naphthol
	0·01	**0·01**	**0·01**	**0·01**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
3	0·95	—	—	—	P	BR	Unknown
	0·91	0·90	0·91	0·91	DR	Y	1-Naphthol
	0·86	0·85	0·86	0·85	O	Y	2-Naphthol
	0·41	—	—	—	P	P	1:2-Dihydroxynaphthalene
	0·31	**0·30**	**0·31**	**0·31**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
4	0·86	—	—	—	P	BR	Unknown
	0·82	0·81	0·82	0·82	DR	Y	1-Naphthol
	0·75	0·75	0·75	0·75	O	Y	2-Naphthol
	0·26	**0·27**	**0·27**	**0·26**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
5	0·66	—	—	—	P	BR	Unknown
	0·60	0·61	0·60	0·60	DR	Y	1-Naphthol
	0·50	0·52	0·50	0·51	O	Y	2-Naphthol
	0·12	**0·10**	**0·11**	**0·10**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
					Butanol extracts		
6	0·95	0·95	—	—	DR	Y	1-Naphthol
	—	—	0·94	0·93	O	Y	2-Naphthol
	—	—	**0·92**	**0·91**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
	0·82	**0·82**	**0·80**	**0·80**	DR	Y	1-Naphthylsulphuric acid
	0·74	**0·74**	**0·74**	**0·73**	P	P	Unknown
	—	**0·28**	**0·29**	**0·28**	DR	Y	1-Naphthyl glucosiduronic acid
	—	—	**0·11**	**0·10**	DR	Y	Unknown*
7	0·91	0·91	—	—	DR	Y	1-Naphthol
	—	—	0·90	0·90	O	Y	2-Naphthol
	—	—	**0·83**	**0·83**	DR	Y	1:2-Dihydronaphthalene-1:2-diol
	0·63	**0·63**	**0·65**	**0·65**	DR	Y	1-Naphthylsulphuric acid
	0·50	**0·50**	**0·49**	**0·50**	P	P	Unknown
	—	**0·12**	**0·12**	**0·11**	DR	Y	1-Naphthyl glucosiduronic acid
	—	—	**0·08**	**0·08**	DR	Y	Unknown*

* Probably 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (see text).

acid and 2-naphthylsulphuric acid corresponding to 1% of the hydrocarbon administered were dissolved in the urine of rats dosed with naphthalene. When butanol extracts of this urine were examined by means of paper chromatography, the two conjugates of 2-naphthol were easily detectable on the chromatograms and could be differentiated from the 1-naphthyl glucosiduronic acid and 1-naphthylsulphuric acid present. These findings suggest that, if 2-naphthyl glucosiduronic acid and 2-naphthylsulphuric acid are excreted by rats dosed with naphthalene, these compounds are present in the urine in amounts corresponding to considerably less than 1% of the dose administered. In later experiments, naphthalene was administered to rabbits and rats at a dose level of 1 g. instead of 0·5 g./kg. body wt. The results obtained, however, were similar to those just described. Moreover, in the case of rats, the animals were found to excrete the same metabolites whether they were dosed by stomach tube or by intraperitoneal injection.

The R_F values of three unidentified compounds are included in Table 1. One of these was detected only in the urine of guinea pigs dosed with naphthalene and the fact that the compound reacted immediately with diazo reagents to give coloured products suggests that it was phenolic in character.

The compound which had R_F value 0·74 in solvent system 6 and 0·50 in solvent system 7 was present in the urine of every species studied and was detected on paper chromatograms only after its decomposition with acid, when colours were given with the diazo reagents which resembled those of 1:2-dihydroxynaphthalene, a finding which suggests that the unknown substance may have been a conjugate of this compound.

The third unknown compound gave a positive test for 1-naphthol after, but not before, it had been treated with acid. Further information concerning the nature of this compound was obtained in the following experiment.

A butanol extract of the urine of rats dosed with naphthalene was prepared by means of the procedure described earlier. A sample of the extract was spotted on a paper strip which was developed in solvent system 6 (see Table 1). After the chromatogram had been developed, the section of the paper strip which carried the unknown metabolite of naphthalene (R_F 0·11) was removed and immersed for 10 min. in warm water (5 ml.). When a sample (2 ml.) of the aqueous extract so prepared was heated in a boiling-water bath for 30 min. in the presence of 3N-HCl and naphthoresorcinol, a strong positive test for glucuronic acid was obtained. The pH of a second sample of the aqueous extract (2 ml.) was adjusted to 1 with conc. HCl and heated in a boiling-water bath for 20 min. The mixture was then cooled to room temperature and extracted with an equal volume of ether. The evaporated ether extract, when examined by paper chromatography, was found to contain both 1- and 2-naphthol. None of the compounds was detected when a second paper strip which had been spotted with a butanol extract of normal rat urine was developed simultaneously with that used in the experiment just described.

The possibility that the third unknown compound was 1- or 2-naphthyl glucosiduronic acid, each of which gives rise to the corresponding naphthol on acid treatment, was excluded by the fact that its R_F values differed significantly from those of these compounds. As will be shown later in this paper, acid decomposition of 1:2-dihydronaphthalene-1:2-diol leads to the formation of 1- and 2-naphthol, and the finding that the unidentified metabolite of naphthalene gave rise to these substances in addition to glucuronic acid is in accordance with the possibility that the compound was a glucuronic acid conjugate of 1:2-dihydronaphthalene-1:2-diol. This, when taken in conjunction with the fact that good evidence has been obtained that the urine of rabbits (Corner, Billett & Young, 1954) and rats (this paper) contains 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid suggests that this compound was the third unidentified substance present on the paper chromatograms.

ISOLATION STUDIES

From the urines of animals of different species dosed with naphthalene a number of metabolites were isolated and identified.

1-*Naphthol*

A solution of naphthalene in arachis oil (20%, w/v) was administered by intraperitoneal injection to 117 male white mice of average body wt. of 20 g. The animals were anaesthetized lightly with ether to facilitate dosing and each animal received 0·5 g. naphthalene/kg. body wt. on each of two successive days. This was repeated on the fourth and fifth days after the first dosing. Some mice died during this experiment, and in all 3·17 g. of naphthalene were administered.

The urine was adjusted to pH 7 and extracted continuously with ether for 20 hr. The ether extracts were combined and when evaporated to dryness yielded a pale brown residue. Water (5 ml.) was added to the material, most of which dissolved on warming. The aqueous solution was cooled to room temperature, brown tarry material was removed by filtration and the aqueous filtrate was extracted with an equal volume of ether. Extraction with ether was repeated four times, and the combined extracts were taken to dryness. A little of the material so obtained was dissolved in water. One half of this solution was treated with diazotized sulphanilic acid followed by excess 2N-NaOH and gave a deep cherry-red colour. The other half was adjusted to pH 1 with dil. HCl and warmed on a water bath for 1 min. When the solution was made alkaline with 2N-NaOH, cooled and treated with diazotized sulphanilic acid a cherry-red colour developed, but this did not appear to be more intense than that given by the first half of the solution. It appeared, therefore, that very little dihydronaphthalenediol was present in the material tested. When a small quantity of the material was treated with diazotized *p*-nitraniline it gave a strong positive test for 1-naphthol.

The residue obtained by evaporation of the ether extracts was heated in water (3 ml.) and was dispersed by rapid stirring until most of it had dissolved. The solution was filtered and the filtrate was left in the refrigerator for 24 hr. The crystals which formed were separated and dried at room temperature by suction on the filter. After the material so obtained was refluxed with boiling light petroleum (b.p. 60–80°) 5 mg. of colourless crystals were obtained, m.p. 94°, mixed m.p. with 1-naphthol, 94–95°. The 1-naphthol isolated by this procedure was obtained from water-soluble material contained in ether extracts of neutralized urine of mice dosed with naphthalene. An additional small quantity of this metabolite was isolated from the water-insoluble brown pigment contained in the ether extracts. The material was refluxed with boiling light petroleum (b.p. 60–80°), and the extracts, when concentrated, yielded 4 mg. of crystalline material, m.p. 94°, mixed m.p. with 1-naphthol, 94–95°.

The compound (8 mg.) was acetylated by acetic anhydride in pyridine and gave 3 mg. of crystalline material, m.p. 48°, mixed m.p. with 1-naphthylacetate, 48–49°. The total yield of 1-naphthol was 9 mg. and accounted for 0·3% of the naphthalene administered to mice.

2-*Naphthol*

Three male rabbits (average body wt. 3 kg.) were dosed with naphthalene as a 20% (w/v) soln. in arachis oil so that each animal received a dose by stomach tube of 1 g./kg. body wt. on each of two successive days. The urine (pH 7·0–7·5) was collected over a 3-day period after the first dosing, filtered through glass wool and continuously extracted with peroxide-free ether for 18 hr. The combined ether extracts were evaporated to dryness. The brown residue so obtained was treated with warm water (8 ml.) and the suspension was freed from insoluble tarry material by filtration. When the filtrate was cooled to room temperature it deposited a small quantity of crystalline material. This was filtered off, recrystallized from water and dried by suction on the filter to yield 20 mg. of colourless crystals, m.p. 121–122°, alone and when mixed with 2-naphthol. The filtrate from this procedure gave a positive test for 1-naphthol with diazotized *p*-nitraniline, but attempts to isolate the metabolite were unsuccessful. The insoluble tarry material obtained in the course of this isolation was extracted for 15 min. with boiling light petroleum (b.p. 60–80°) under reflux, and when the extract was concentrated at room temperature in a current of air crystalline material was deposited. This was filtered off and recrystallized once from hot water and from aqueous ethanol to give 87 mg. of pinkish crystals, m.p. 121–122°, alone and when mixed with 2-naphthol. (Found: C, 83·4; H, 5·7. Calc. for $C_{10}H_8O$: C, 83·3; H, 5·6%.)

When 38 mg. of the metabolite were acetylated by acetic anhydride in pyridine, 24 mg. of a colourless crystalline derivative were obtained, m.p. 70–70·5° (alone and when mixed with 2-naphthyl acetate). (Found: C, 77·3; H, 5·2. Calc. for $C_{12}H_{10}O_2$: C, 77·4; H, 5·4%.)

In this experiment the amount of 2-naphthol which was isolated corresponded to 0·5% of the naphthalene administered to the rabbits.

1:2-*Dihydronaphthalene*-1:2-*diol*

Ten male guinea pigs of average body wt. 0·65 kg. were dosed intraperitoneally on each of two successive days with a solution of naphthalene in arachis oil (20%, w/v) so that each animal received 0·5 g./kg. body wt. at each dosing.

Ether extracts of the neutralized urine were taken to dryness and gave a reddish brown residue. Attempts to isolate naphthol from this residue by means of the procedure described earlier were unsuccessful, but the presence in the residue of dihydronaphthalenediol was established as follows. The residue was treated with warm water (200 ml.) and insoluble tarry material was removed by filtering. The reddish yellow filtrate was cooled to room temperature and was then extracted continuously with peroxide-free ether for 24 hr. The ether extract was evaporated to dryness and the red, semi-crystalline material so obtained was dried *in vacuo*. This material gave a faint pink colour with diazotized sulphanilic acid in the presence of excess 2N-NaOH. After it was heated in acid solution for 1 min., however, the material gave a deep cherry-red colour with the alkaline diazo reagent. These findings implied that the material contained a trace of naphthol and a considerable amount of a compound which gives naphthol after acid treatment.

The red residue was crystallized once from *cyclo*hexane and 0·308 g. of pink crystals were obtained, m.p. 120–122°. The compound was recrystallized once more from *cyclo*hexane and twice from benzene. The final yield of colourless crystalline material was 0·104 g., m.p. 126–127°, $[\alpha]_D^{20}$ – 157° in ethanol (*c*, 1). (Found: C, 74·0; H, 6·1. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%.) The compound was acetylated by acetic anhydride in pyridine and yielded a colourless crystalline derivative, m.p. 77–78°, $[\alpha]_D^{22}$ – 434° in ethanol (*c*, 1). (Found: C, 68·8; H, 6·2. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.) Young (1947) has reported the isolation of laevorotatory 1:2-dihydronaphthalene-1:2-diol from the urine of rats dosed with naphthalene, m.p. 127–128°, $[\alpha]_D^{20}$ – 154° in ethanol (*c*, 1). The diacetate of this compound melted at 78–79° and showed $[\alpha]_D^{20}$ – 423° in ethanol (*c*, 1). Cook, Loudon & Williamson (1948, 1950) and Booth & Boyland (1949) have shown that this metabolite has the *trans* configuration.

The properties of the metabolite isolated from the urine of guinea pigs indicate that these animals, like certain strains of rat, are able to convert naphthalene into (–)-*trans*-1:2-dihydronaphthalene-1:2-diol. This was confirmed by the finding that the m.p. was not depressed when the compound isolated from guinea pig urine was mixed with the (–)-*trans*-1:2-dihydronaphthalene-1:2-diol isolated from rat urine by Young (1947). The amount of pure dihydronaphthalenediol isolated corresponded to 1·2% of the naphthalene administered to guinea pigs. In two further experiments, the amounts of the metabolite isolated accounted for 0·8 and 1·1% of the hydrocarbon injected.

The recrystallized compound was examined by paper chromatography and was found to be free from naphthol. After the compound had been heated in acid solution, however, chromatographic studies showed that the formation of 1- and 2-naphthol had occurred, with 1-naphthol preponderating. In later experiments it was found that, in this respect, optically inactive and dextrorotatory 1:2-dihydronaphthalene-1:2-diol behave similarly to the metabolite isolated from guinea pig urine. Recently, Boyland & Sims (1953) have reported that synthetic (±)-*trans*-1:2-dihydronaphthalene-1:2-diol yields 1- and 2-naphthol on acid decomposition.

1:2-*Dihydro*-2-*hydroxy*-1-*naphthyl glucosiduronic acid*

Corner *et al.* (1954) have described the separation of a glucosiduronic acid fraction from the urine of rabbits dosed with naphthalene, and have established that this fraction contains a glucuronic acid conjugate of dextrorotatory 1:2-dihydronaphthalene-1:2-diol. In the following experiments, evidence has been obtained suggesting that rats also convert naphthalene into 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid. Experiments with guinea pigs and mice have yielded no evidence that this compound is formed from naphthalene in either of these species.

Twenty-four male hooded rats (average body wt. 320 g.) were dosed once on each of two successive days with a solution of naphthalene in arachis oil (20%, w/v). Each animal received 1 g. naphthalene/kg. body wt. by stomach tube at each dosing. Urine (pH 6·5–7·0) was collected for 3 days after the first dosing, filtered, extracted with ether and evaporated under reduced pressure to one-fifth of its volume. A glucosiduronic acid fraction in the form of a brown hygroscopic powder (19 g.) was separated from the concentrate by the method of Corner *et al.* (1954). The fraction was washed thoroughly with ethanol and again with ether and when it was examined by means of paper chromatography it was found to contain traces of 1-naphthyl

glucosiduronic acid, and a compound with R_F value 0·11 in solvent system 6 and R_F value 0·08 in solvent system 7 (see Table 1). Evidence which suggests that this compound is a glucuronic acid conjugate of dihydronaphthalenediol has been described earlier in this paper and this possibility was tested further as follows.

Isolation of 1-naphthyl glucosiduronic acid after acid treatment of the glucosiduronic acid fraction from rat urine. When a sample of the fraction (3 g.) at pH 6–7 was treated with *p*-toluidine hydrochloride under conditions favourable to the precipitation of 1- and 2-naphthyl glucosiduronic acid (cf. Berenbom & Young, 1951) no precipitate was obtained. This was not unexpected as examination of the fraction by paper chromatography had revealed that 1-naphthyl glucosiduronic acid was present only in traces. After a second sample of the fraction (3 g.) had been heated at pH 1 for 5 min. in a boiling-water bath, and adjusted to pH 6–7, treatment of the mixture with *p*-toluidine hydrochloride (0·3 g.) led to the precipitation of brown crystalline material. This was separated and dissolved in hot water, and the solution was treated with animal charcoal. Charcoal was removed by filtration and the yellow filtrate deposited silky white needles when cooled to room temperature. The precipitate, when recrystallized once from water and dried over P_2O_5 *in vacuo*, gave 0·160 g. of white crystalline material. When 0·150 g. of this material was dissolved in 6N-HCl (1·5 ml.) and the solution cooled to 4°, a crystalline precipitate was formed. This was recrystallized once from water to yield 0·077 g. of colourless needles, $[\alpha]_D^{20} - 87°$ in ethanol (*c*, 0·5), m.p. 198–199°, alone and when mixed with 1-naphthyl glucosiduronic acid. The compound gave positive tests for 1-naphthol and glucuronic acid after it was heated in acid solution. The finding that the glucosiduronic acid fraction yielded 1-naphthyl glucosiduronic acid when treated with acid is consistent with the view that the fraction contained 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid.

Acid hydrolysis of the glucosiduronic acids present in the fraction. A sample of the fraction (0·1 g.) was dissolved in water (2 ml.) and the solution was adjusted to pH 1 with conc. HCl and heated for 1 hr. in a boiling-water bath. The mixture was cooled to room temperature, adjusted to pH 6–7 with 2N-NaOH and extracted once with an equal volume of ether. The ether extract was evaporated to dryness and the reddish yellow residue so obtained was examined by means of paper chromatography for the presence of hydroxy derivatives of naphthalene. The results showed that 1-naphthol was the major product of the hydrolysis and that small amounts of 2-naphthol were also formed. Neither 1:2- nor 1:4-dihydroxynaphthalene could be detected. It is now known that the acid decomposition of 1:2-dihydronaphthalene-1:2-diol leads to the formation of both 1- and 2-naphthol, and it seems probable that a similar change would occur after acid treatment of 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid.

Isolation of 1:2-dihydronaphthalene-1:2-diol after treatment of the glucosiduronic acid fraction with β-glucuronidase. By means of paper chromatography it was found that the glucosiduronic acid fraction contained neither dihydronaphthalenediol nor naphthol in the free state. A solution of the powder (3 g.) in 0·2M acetate buffer (pH 5·2) was incubated with β-glucuronidase (prepared by the method of Kerr & Levvy, 1951), at 37° for 2 hr. The mixture was then filtered, the filtrate was adjusted to pH 8 with 2N-NaOH and extracted with ether for 24 hr. The ether extracts were evaporated to dryness and gave a pink crystalline residue. This was examined by paper chromatography and was found to contain a trace of 1-naphthol and a considerable amount of dihydronaphthalenediol. 1:2-Dihydroxynaphthalene and 2-naphthol were not detected in this residue. The material was recrystallized once from *cyclo*hexane and again from benzene and gave 0·092 g. colourless silky needles, m.p. 104–105°, $[\alpha]_D^{20} - 27°$ in ethanol (*c*, 1). (Found: C, 73·6; H, 6·4. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%.) The compound was examined by means of paper chromatography and was found to be free from naphthol. 1-Naphthol and traces of 2-naphthol were formed, however, when the compound was heated in acid solution. The compound was acetylated by acetic anhydride in pyridine and the product melted at 74–75° and showed $[\alpha]_D^{19} - 88°$ in ethanol (*c*, 0·5). (Found: C, 68·8; H, 5·8. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.)

A second experiment was carried out in which β-glucuronidase was allowed to act on a glucosiduronic acid fraction. This fraction, however, was isolated from the urine of rats dosed with naphthalene by intraperitoneal injection. After the powder (3 g.) was treated with β-glucuronidase under the conditions just described, 0·148 g. of crystalline material, m.p. 102–103°, $[\alpha]_D^{22} - 5°$ in ethanol (*c*, 1) was obtained. (Found: C, 74·3; H, 6·2. Calc. for $C_{10}H_{10}O_2$: C, 74·0; H, 6·2%.) The material was acetylated and gave a crystalline derivative, m.p. 74–75°, $[\alpha]_D^{22} - 18°$ in ethanol (*c*, 0·5). (Found: C, 67·9; H, 5·7. Calc. for $C_{14}H_{14}O_4$: C, 68·3; H, 5·7%.)

It is apparent, therefore, that treatment of the glucosiduronic acid fraction with β-glucuronidase led to the liberation of 1:2-dihydronaphthalene-1:2-diol. The melting point and specific rotation of the compound, and those of its diacetate, show that the compound was a mixture of laevo- and dextro-rotatory 1:2-dihydronaphthalene-1:2-diol, in which the former preponderated. This was so whether naphthalene was administered to male hooded rats by stomach tube or by intraperitoneal injection, although the products obtained showed different specific rotations.

The results of the experiments just reported show that a glucosiduronic acid fraction obtained from the urine of rats dosed with naphthalene, when decomposed by acid, gives rise to 1-naphthyl glucosiduronic acid, 1-naphthol and 2-naphthol. Furthermore, it yields 1:2-dihydronaphthalene-1:2-diol when acted upon by β-glucuronidase. These findings support the view that the rat, like the rabbit (Corner *et al.* 1954), is able to convert naphthalene into 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid.

1-*Naphthylmercapturic acid*

Young (1947) has described a method for the isolation of 1-naphthylmercapturic acid from the urine of rats dosed with naphthalene. In the present work the application of this method to the urines of mice and guinea pigs dosed with naphthalene has revealed that formation of this mercapturic acid occurs also in these species.

After 1·6 g. of naphthalene had been administered to mice by intraperitoneal injection, 0·267 g. of crude 1-naphthylmercapturic acid, m.p. 167°, was isolated from $CHCl_3$ extracts of the acidified urine. This corresponded to 6% of the naphthalene administered. The material was purified by conversion into the sodium salt (cf. Bourne & Young, 1934), which was then treated with conc. HCl to yield 0·236 g. of 1-naphthylmercapturic acid. This was recrystallized once from aqueous ethanol and gave a product,

m.p. 170–170·5°, alone and when mixed with an authentic sample of 1-naphthylmercapturic acid. (Found: C, 62·6; H, 5·3; S, 11·5. Calc. for $C_{15}H_{15}O_3NS$: C, 62·3; H, 5·2; S, 11·1%.)

A much smaller amount of the mercapturic acid (0·037 g.) was isolated from the urine of guinea pigs dosed intraperitoneally with a total of 2·2 g. naphthalene. After one recrystallization from ethanol and water the compound had m.p. 169–170°, unchanged by admixture with an authentic sample of 1-naphthylmercapturic acid. (Found: C, 62·4; H, 5·2; S, 11·3. Calc. for $C_{15}H_{15}O_3NS$: C, 62·3; H, 5·2; S, 11·1%.) The amount of the metabolite isolated corresponded to 0·6% of the naphthalene administered.

DISCUSSION

The results of the present investigation are summarized in Fig. 1. Hitherto the only evidence available for the presence of unconjugated 1-naphthol in the urines of animals dosed with

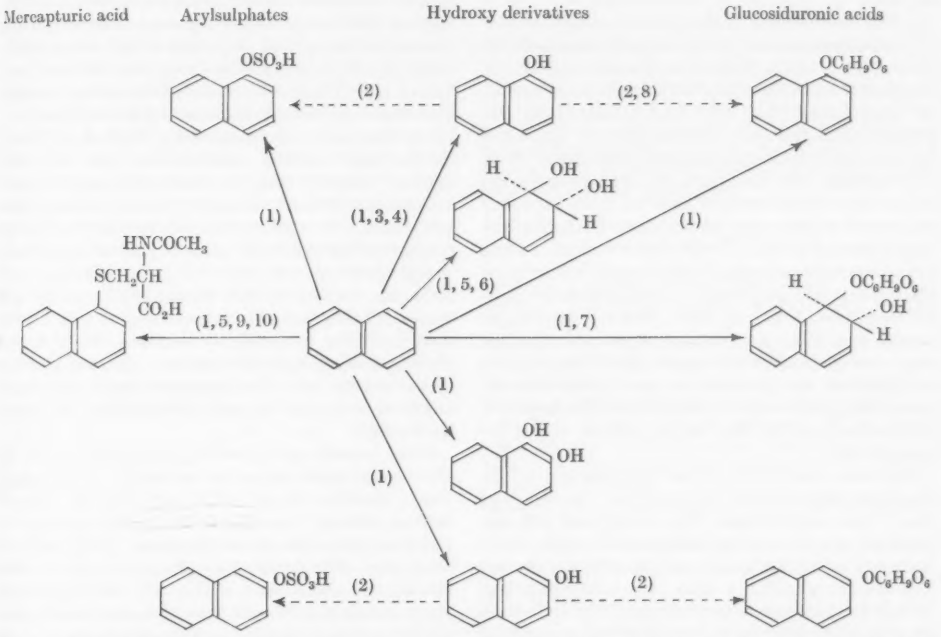

Fig. 1. The metabolism of naphthalene in animals of different species. Reactions undergone by 1- and 2-naphthol *in vivo* have been included and are indicated by broken arrows. Figures on arrows refer to references given below. The presence of the compounds in urines of different species is indicated in the table below by +; compounds not detected are indicated by –:

Compound studied	Rat	Rabbit	Guinea pig	Mouse
1-Naphthol	+[1]	+[1, 3, 4]	+[1]	+[1]
1-Naphthylsulphuric acid	+[1]	+[1]	+[1]	+[1]
1-Naphthyl glucosiduronic acid	+[1]	+[1]	–[1]	+[1]
2-Naphthol	+[1]	+[1]	+[1]	+[1]
2-Naphthylsulphuric acid	–[1]	–[1]	–[1]	–[1]
2-Naphthyl glucosiduronic acid	–[1]	–[1]	–[1]	–[1]
1:2-Dihydroxynaphthalene	–[1]	–[1]	+[1]	–[1]
1:2-Dihydronaphthalene-1:2-diol	+[1, 5]	+[6]	+[1]	+[1]
1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid	+[1]	+[7]	–[1]	–[1]
1-Naphthylmercapturic acid	+[5, 9]	+[10]	+[1]	+[1]

[1] This paper.
[2] Berenbom & Young (1951).
[3] Igersheimer & Rubin (1910).
[4] Adams (1930).
[5] Young (1947).
[6] Booth & Boyland (1949).
[7] Corner *et al.* (1954).
[8] Lesnik & Nencki (1886).
[9] Stekol (1935).
[10] Bourne & Young (1934).

naphthalene has been based on the results of non-specific colour tests (Igersheimer & Rubin, 1910; Adams, 1930). In the present work the excretion of 1- and 2-naphthol by rabbits, rats, mice and guinea pigs dosed with naphthalene has been shown by means of paper chromatography. In the case of 1-naphthol in mouse urine and 2-naphthol in rabbit urine, the findings have been confirmed by the isolation of the naphthol. 2-Naphthol has not previously been reported as a metabolite of naphthalene.

It has been shown in the present work that the acid decomposition of 1:2-dihydronaphthalene-1:2-diol leads to the formation of small amounts of 2-naphthol in addition to 1-naphthol, and it should be emphasized that our demonstration of the presence of naphthols in the free state in the urines did not involve the use of acid conditions. It is most unlikely, therefore, that the naphthols present in the urine were artifacts derived from naphthol conjugates or from free or conjugated 1:2-dihydronaphthalene-1:2-diol. It is known that, when administered to animals, 1- and 2-naphthol undergo conjugation with sulphuric acid and glucuronic acid (cf. Berenbom & Young, 1951), and it is interesting to note that although 1- and 2-naphthol in the free state were present in the urines of all four species, evidence for the presence of conjugates was obtained only in the case of 1-naphthol. This aspect of naphthalene metabolism is at present under investigation.

Evidence was obtained for the presence of 1:2-dihydroxynaphthalene in the urine of guinea pigs dosed with naphthalene. This compound was not detected, however, in the urines of any of the other species studied, although our experiments do not exclude the possibility that this compound was present in these urines in conjugated form. There is some doubt concerning the origin of this compound. It might arise by hydroxylation of naphthalene or naphthol, or it might be formed by dehydrogenation of 1:2-dihydronaphthalene-1:2-diol. Another possibility is that the compound arises from the interaction of 1:2-naphthoquinone and amino acids, a reaction which is known to give rise to red amino acid derivatives together with 1:2-dihydroxynaphthalene. It is noteworthy that naphthalene urines show a red colour and that this is especially intense in the case of guinea pig urine. 1:2-Naphthoquinone is a possible metabolite of naphthalene and its presence in the urines of naphthalene-dosed animals has been reported by Penzoldt (1886).

Evidence has been obtained that all four species studied excrete 1:2-dihydronaphthalene-1:2-diol. Marked differences are encountered, however, with respect to the optical activities of the compounds obtained. This metabolite was first isolated by Young (1947) in the form of a laevorotatory isomer from the urine of white rats dosed with naphthalene. When this work was repeated by Booth & Boyland (1949) these workers isolated from rat urine an optically inactive form of 1:2-dihydronaphthalene-1:2-diol in addition to the laevorotatory form of this compound. The properties of the laevorotatory compound isolated by Booth & Boyland showed that it was identical with that obtained by Young (see Table 2). In the present work only optically inactive 1:2-dihydronaphthalene-1:2-diol was separated from the urine of hooded rats, whereas the same procedure applied to guinea pig urine yielded laevorotatory 1:2 - dihydronaphthalene - 1:2 - diol, identical with that originally isolated from rat urine by Young.

Some interest attaches to the optical activity of the dihydronaphthalenediol excreted in conjugated form. Rabbits dosed with naphthalene excrete dextrorotatory 1:2-dihydronaphthalene-1:2-diol in the free state (Booth & Boyland, 1949) and its conjugate 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid (Corner *et al.* 1954). In the present work it has been found that glucuronic acid conjugates of both laevo- and dextro-rotatory 1:2-dihydronaphthalene-1:2-diol are present in the urine of hooded rats dosed with naphthalene, with the glucuronic acid conjugate of the laevorotatory isomer preponderating.

Table 2. *Physical properties of* 1:2-*dihydronaphthalene*-1:2-*diol excreted either in the free state or in conjugation with glucuronic acid in the urines of animals dosed with naphthalene*

	Unconjugated					Conjugated	
	Rats			Rabbits		Rabbits	Rats
Reference	Young (1947)	Booth & Boyland (1949)	This paper	Booth & Boyland (1949)	Guinea pigs This paper	Corner *et al.* (1954)	This paper
Dihydrodiol							
m.p. (°)	127–128	126 101	100	125 101	126–127	127–128	104–105
$[\alpha]_D$ (°)	−154	−158 0	0	+159 0	−157	+157	−27
Diacetate							
m.p. (°)	78–79	77 73	74	77 73	77–78	77	74–75
$[\alpha]_D$ (°)	−423	−436 0	0	+437 0	−434	+421	−88

It is unlikely that racemization of 1:2-dihydronaphthalene-1:2-diol occurred during the isolation of this metabolite, for in the present work it was found that with guinea pig urine the procedure used yielded the laevorotatory form, whereas with rat urine the same procedure led to the isolation of an optically inactive compound. Furthermore, it should be noted that although attempts have been made to racemize the metabolite experimentally, this has not been achieved (cf. Booth & Boyland, 1949) and differences in the optical activity of 1:2-dihydronaphthalene-1:2-diol are therefore the result of processes as yet unexplained. In this connexion it is interesting to note that Wood & Gunsalus (1951) have demonstrated that both D- and L-alanine can be converted into a racemic mixture by dried cells and by partially fractionated cell extracts of *Streptococcus faecalis*. These authors have termed the enzyme concerned in this reaction alanine 'racemase' and have reported that the presence of pyridoxal phosphate is also necessary for these changes to occur. Although the enzyme appears to be specific for alanine, these findings nevertheless draw attention to the possibility that racemization of 1:2-dihydronaphthalene-1:2-diol may occur as the result of an enzymic process.

The conversion of naphthalene into 1-naphthylmercapturic acid has been shown to occur in every species in which this reaction has been studied, namely, rabbits (Bourne & Young, 1934), rats (Stekol, 1935; Young, 1947), dogs (Stekol, 1935), pigs (Stekol, 1936), guinea pigs and mice (this paper). In this respect, as in others already discussed, the effect of species differences on the metabolism of naphthalene is of a quantitative rather than a qualitative nature. The one outstanding difference so far revealed lies in the optical activity of the 1:2-dihydronaphthalene-1:2-diol present in the urines of different species.

SUMMARY

1. A study has been made of the metabolism of naphthalene in rabbits, rats, guinea pigs and mice. Various metabolites of naphthalene have been shown to be present in the urine of the dosed animals by means of paper chromatography or by isolation.

2. All four species have been found to convert naphthalene into 1- and 2-naphthol, 1:2-dihydronaphthalene-1:2-diol, 1-naphthylsulphuric acid (1-naphthyl hydrogen sulphate) and 1-naphthylmercapturic acid.

3. 1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid and 1-naphthyl glucosiduronic acid have been shown to be present in the urines of rabbits and rats dosed with naphthalene. The latter compound has been found to be a metabolite of naphthalene in the mouse.

4. By means of paper chromatography, 1:2-dihydroxynaphthalene has been detected in the urine of guinea pigs dosed with naphthalene.

5. The main difference encountered in the metabolism of naphthalene in animals of different species was in the optical activity of the 1:2-dihydronaphthalene-1:2-diol present in the urine.

This work has been aided by a grant from the Endowment Fund of St Thomas's Hospital. The elementary microanalyses were carried out by Drs Weiler and Strauss. A preliminary account of the work was presented at a meeting of the Biochemical Society held at Bristol on 15 May 1954 (Corner & Young, 1954).

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Some Properties of Human and other Synovial Fluids

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(*Received* 28 *June* 1954)

The main object of this paper is to report measurements made on a small series of human synovial fluids and on a few sheep and ox fluids. These measurements were made in order to test the two methods suggested by Ogston & Stanier (1953*a*) for detecting differences of the specific properties of the hyaluronic acid as it occurs in synovial fluids. These methods, both of which are based on viscosity measurements, were suggested because (1) the viscosity of synovial fluid depends almost entirely on its hyaluronic acid content; (2) the viscosity and particularly its variation with velocity gradient are sensitive to changes in the state of hyaluronic acid. Because the viscous properties vary rapidly with concentration it is necessary either to compare relationships which are independent of concentration or to measure and make allowance for the concentration. In Ogston & Stanier's first method, the ratios of viscosities at zero velocity gradient and at 50 sec.$^{-1}$ are plotted against the viscosities at zero velocity gradient; while dilutions of a given sample of hyaluronic acid give points lying reproducibly on a single curve, different hyaluronic acids give different curves. Scott Blair, Williams, Fletcher & Markham (1954) have proposed a similar method. In their second method, the concentration of hyaluronic acid forms the basis for comparing viscosities, which may be measured under any stated conditions of flow.

Sedimentation measurements were also made on a few representative fluids, in order to obtain further information on the state of their hyaluronic acid. It is concluded (i) that there are inter-species and possibly intra-species differences of the hyaluronic acid of synovial fluid; (ii) that these differences are detectable by comparing viscosities in a way not requiring measurement of concentration; (iii) that the measurement of the concentration of hyaluronic acid in human fluids presents difficulties which make comparisons of viscosity on a concentration basis of doubtful value. Having reached these conclusions, we intend, at least for the present, to discontinue this work.

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EXPERIMENTAL

Synovial fluids. Human 'traumatic' fluids were supplied by Mr R. G. Taylor of the Wingfield-Morris Orthopaedic Hospital, Oxford. They were taken at operation from cases of moderate trauma of the knee joint, free from any obvious disease. Fluids containing blood were not used. The volumes of the fluid varied with the state of the joint from *ca.* 1 ml. to *ca.* 20 ml. The fluids were bottled with full sterile precautions and were stored at 1°. It was usual for a clot, presumably of fibrin, to form within 24 hr. of collection; this was centrifuged off before use, and the separated supernatant fluid subsequently remained clear. Normally all work on a fluid was completed within 3 days of opening the bottle; no sign of infection or degradation was observed within this period. In a few cases, measurements were repeated after several months' storage, and showed no detectable change of properties. A single sample of fluid taken from a normal joint, about 12 hr. *post mortem*, was obtained. Human pathological fluids were supplied by the Rheumatism Research Unit, Canadian War Memorial Hospital, Taplow. Ox fluids were obtained from the slaughterhouse, as described by Ogston & Stanier (1950); sheep fluids were obtained by the same method, either from the slaughterhouse or from sheep used for experiments at the Nuffield Institute for Medical Research, Oxford; in both cases fluid was taken from the ankle (astragalotibial) joint.

Concentration of hyaluronic acid. A mucin clot was formed by acidification of 0·5–1 ml. of fluid and its dry weight determined, after thorough washing, as described by Ogston & Stanier (1950). The adequacy of the washing procedure was checked. In some cases, where enough material was available, duplicate determinations were made; these agreed within 10%. Dilution of the fluid up to threefold, before precipitation was performed, did not seem to affect the result. Dilutions of the fluids were made by weight with the buffer used by Ogston & Stanier (1950) (0·2 M-NaCl, 0·0077 M-Na_2HPO_4, 0·0023 M-KH_2PO_4).

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge and sedimentation coefficients were calculated by the method of Cecil & Ogston (1948). Measurements were made on successive dilutions by about 1·5-fold, until the hyaluronic acid boundary could be no longer observed.

Viscosity was measured at 25° in the Couette viscometer described by Ogston & Stanier (1953*b*), modified to allow measurements to be made on only 2 ml. of fluid. A smaller pot, internal diameter 1·121 cm. was made to fit into the standard pot, and a smaller bob was used, diameter 1·00 cm. × length 4 cm. The annular gap was 0·0605 cm. The accuracy of this instrument was somewhat less than

with the 10 ml. pot, the error of a single measurement being about 3%. Measurements were made in most instances on the original fluid or (if its volume was insufficient) on a dilution of it. In some cases, measurements were made on a series of dilutions, to allow the intrinsic viscosity to be estimated. Each solution was measured at a number of low velocity gradients, the viscosity at zero velocity gradient being obtained by extrapolating $1/\eta_{rel.}$ against velocity gradient; also the viscosity at velocity gradient 50 sec.$^{-1}$ was estimated by interpolation.

RESULTS

Viscosities. Table 1 gives values of log η_0 (η_0 = relative viscosity at zero velocity gradient), log η_{50} (η_{50} = relative viscosity at 50 sec.$^{-1}$), and log η_0/η_{50}. The hyaluronic acid content is given as mg. mucin/ml. These values apply to undiluted human fluids, except where dilution was necessary because of the small volume available. Where dilution has been performed, the dilution factor is given. Serial dilutions were made on ox and sheep fluids. In Fig. 1, log η_0/η_{50} is plotted against log η_0; this is a log version of Ogston & Stanier's (1953*a*) plot, having the advantage of less extended scales. In Fig. 2, log η_0 is plotted against mg. mucin/ml.

Intrinsic viscosities ($[\eta]_c = (\eta_{rel.} - 1)/c$, $c = 0$). These were estimated by extrapolating log $\{(\eta_{rel.} - 1)/c\}$ against c to zero concentration. In each case, two plots were made, using values of $\eta_{rel.}$ at velocity gradients 0 and 50 sec.$^{-1}$ respectively; these converged to the same intercept at $c = 0$ within 0·1 log units. Values are given in Table 2, c being expressed as g. hyaluronic acid complex/100 ml.; the assumption is made that human mucin is like ox mucin (Ogston & Stanier, 1952) in containing 45% of the complex by weight.

Sedimentation coefficients. The observed values of S were first corrected to water at 20°. Correction was then applied for the effect of protein on the sedimentation of hyaluronic acid by use of the empirical relationship found by Johnston (1955*a*),

$$(1/S)_{corrected} = (1/S)_{observed} - 0{\cdot}043P,$$

where P is the concentration of protein in g./100 ml., estimated from the sedimentation schlieren diagram. $1/S_{20}$ was then plotted against c (g. hyaluronic acid complex/100 ml.). From these plots values of S_0 (S_{20} at zero concentration) and $d(1/S)/dc$ were obtained, and are given in Table 2.

DISCUSSION

Expression of concentration. All the measurements of concentration have been based on the dry weight of mucin clot. This concentration scale is suitable for the comparison of properties of different synovial fluids and for the extrapolation of S to zero concentration. For the calculation of $[\eta]_c$ and $d(1/S)/dc$, and of quantities derived from them, the concentration of hyaluronic acid, together with any material with which it is associated in solution, is required. It has been established that ox 'hyaluronic acid' is a complex containing protein, and that this complex forms 45% by weight of mucin. Although similar information is not available for other 'hyaluronic acids', it seems likely that these also occur as a similar complex; this assumption has been made and the term 'hyaluronic acid' is used in this sense.

Non-Newtonian viscosity. The plot of log η_0/η_{50} against log η_0 (Fig. 1) gives a measure of the relationship of the variation of viscosity with velocity gradient to the viscosity at zero velocity gradient. Ox, sheep and human fluids are clearly different from each other; and there is a suggestion that rheumatoid arthritis fluids differ from other human fluids, both traumatic and pathological.

The deviations, amongst the human fluids, from any common curve are outside the experimental errors and this shows that the properties of the hyaluronic acids in different traumatic fluids vary (cf. Johnston, 1955*b*). It must be remembered that all these fluids were taken from joints which had suffered varying degrees of trauma; this may have affected the hyaluronic acid, but the series is too small to make any attempt to correlate the degree of trauma and the viscous properties. It is interesting that the single sample obtained *post mortem* gives a point lying well off the mean curve of traumatic fluids.

Viscosity and concentration. Fig. 2 again shows clear differences between ox, sheep and human fluids, but the points for the human fluids are very much more scattered than those in Fig. 1. Little of this scatter can be due to errors of determining either η_0 or the mucin content of a given fluid. It must therefore be ascribed, either to variation from fluid to fluid of the viscosity due to a given concentration of hyaluronic acid (if the mucin content measures this correctly); or to variation from fluid to fluid of the proportion of hyaluronic acid in mucin. Of these possibilities, the second seems the more likely, and might be caused by variations of the qualities or amounts of protein or ionic constituents of the fluids (cf. Sundblad, 1950). Whatever the cause of this variation, it does not seem seriously to affect the relationship between viscosity and its variation with velocity gradient; one must conclude that while this relationship forms a suitable basis for comparing the specific properties of hyaluronic acids in synovial fluids, the comparison of viscosity with mucin content does not.

Sedimentation coefficients and intrinsic viscosities. The values both of $d(1/S)/dc$ and of $[\eta]_c$ for human hyaluronic acids, given in Table 2, are affected by the uncertainty about the estimation of concentration by mucin clot formation, noted above. These

Table 1 (*a*). *Concentrations and relative viscosities of synovial fluids from human joints*

Fluid	Dilution	Concentration (mg. mucin/ml.)	log η_0	log η_{50}	log η_0/η_{50}
Human fluids from traumatized knees					
Tr	1/7·77	1·54	0·73	0·63	0·094
Ta	—	8·3	2·48	1·80	0·68
Mi	1/1·82	5·8	1·55	1·28	0·28
Mi	1/3	3·5	1·10	0·95	0·14
Sa	—	6·4	1·37	1·12	0·25
Sh	—	8·3	2·09	1·51	0·58
Mo	—	5·9	1·18	0·97	0·21
Ba	1/2·06	6·7	2·46	1·68	0·78
Bul	—	5·8	1·74	1·33	0·41
Bur	—	10·0	1·74	1·45	0·30
Wo	—	8·6	2·16	1·60	0·56
Va	1/1·59	5·2	2·05	1·45	0·60
Pev	1/2	6·6	2·12	1·54	0·58
Du	1/2	—	1·39	1·18	0·21
Pathological human fluids					
Br (1)	Rheumatoid arthritis	—	4·9	1·64	1·36	0·28
(2)		—	9·4	2·17	1·61	0·56
(3)		—	6·2	1·31	1·20	0·11
(4)		—	5·5	1·71	1·41	0·30
(5)		—	6·9	2·03	1·58	0·44
(6)		—	5·2	1·44	1·25	0·19
(7)		1/1·28	5·9	1·54	1·31	0·23
Pe		—	5·3	2·13	1·59	0·54
La, Rheumatoid arthritis, untreated		—	—	0·92	0·86	0·06
La, after cortisone		1/2·61	—	0·88	0·80	0·08
Wa, Disseminated lupus erythematosis, treated cortisone		—	—	0·89	0·82	0·07
Coo, Reiter's syndrome, untreated		—	4·3	1·84	1·36	0·48
Cor, Pulmonary osteoarthropathy, untreated		—	6·1	2·11	1·53	0·58
Ja, Osteoarthritis		—	7·0	2·56	1·72	0·84
Post-mortem fluid (normal)		—	4·7	2·48	1·53	0·95

Table 1 (*b*). *Concentrations and relative viscosities of synovial fluid from joints of sheep and ox*

Fluid	Dilution	Concentration (mg. mucin/ml.)	log η_0	log η_{50}	log η_0/η_{50}
Sheep ankle fluids					
A	—	5·8	3·14	1·70	1·44
B (1)	—	5·0	2·33	1·42	0·91
(2)	1/1·66	3·0	1·57	1·09	0·48
C (1)	—	5·4	2·50	1·52	0·98
(2)	1/1·62	3·4	1·73	1·19	0·54
(3)	1/1·95	2·8	1·46	1·06	0·40
(4)	1/3·30	1·65	0·98	0·79	0·19
Ox joint fluids					
Ankle fluid 64 (1)	—	2·04	1·21	0·82	0·39
(2)	1/1·47	1·38	0·86	0·63	0·23
(3)	1/2·01	1·01	0·68	0·51	0·17
Ankle fluid 65 (1)	—	4·93	3·00	1·50	1·50
(2)	1/1·31	3·75	2·29	1·31	0·98
(3)	1/1·80	2·73	1·73	1·10	0·63
Ankle fluid 61	—	2·22	1·54	1·02	0·52
Knee fluid 29*K* (1)	1/1·70	2·60	1·70	0·99	0·71
(2)	1/2·56	1·73	1·21	0·80	0·41
(3)	1/5·1	0·87	0·77	0·51	0·26
(4)	1/10·3	0·43	0·44	0·30	0·14
(5)	1/20·5	0·22	0·14	0·09	0·05
Ankle fluid 37*A* (1)	—	1·82	1·54	0·98	0·56
(2)	1/1·50	1·21	1·12	0·78	0·34
(3)	1/2·25	0·81	0·82	0·60	0·22

Table 2. *Sedimentation coefficients, intrinsic viscosities, and molecular dimensions of hyaluronic acids*

Fluid	$10^{13} \times S_0$	$10^{-13} \times$ d(1/S)/dc	$[\eta]_c$	$10^{-6} \times M$	V'	J
Human, traumatic						
Bul	8·7	2·0	17	2·6	195	7
Bur	6·1	1·1	13	1·2	61	14
Wo	5·4	1·5	22	1·2	68	18
Va	5·2	1·3	36	1·3	120	30
Pe	9·5	2·3	30	3·7	220	10
Br (4) Rheumatoid arthritis	6·7	2·6	17	1·7	195	7
Sheep ankle fluid (*C*)	11·5	2·6	35	5·5	320	8
Ox	14·7*	3·3*	55*	10	520	8

* From Ogston & Stanier (1952).

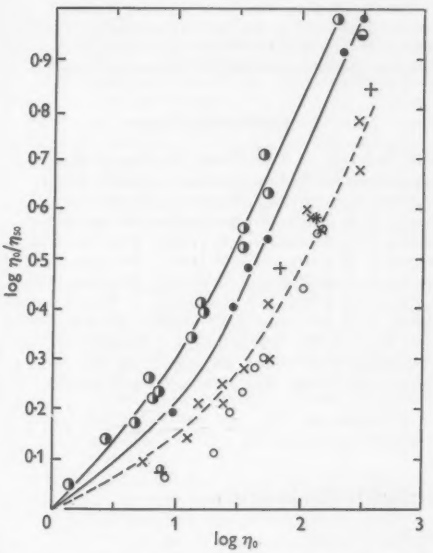

Fig. 1. Ordinate log η_0/η_{50}, abscissa log η_0 (see text) for various synovial fluids, plotted from Table 1. ◑, Ox; ●, sheep; ×, human traumatic; ○, human rheumatoid arthritis; +, other human pathological fluids; ◒, normal human fluid obtained *post mortem*.

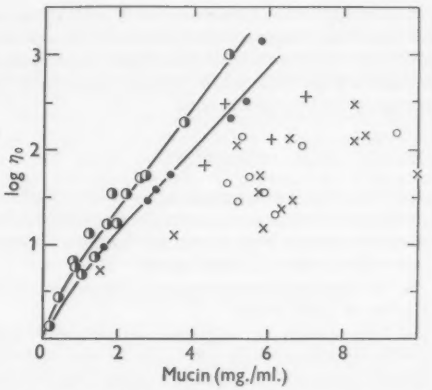

Fig. 2. Ordinate log η_0 (see text); abscissa concentration in mg. mucin/ml. for various synovial fluids, plotted from Table 1. ◑, Ox; ●, sheep; ×, human traumatic; ○, human rheumatoid arthritis; +, other human pathological fluids.

values are therefore less reliable than those for ox and sheep hyaluronic acids. The values of S_0 for sheep and human hyaluronic acids may be affected by the high protein content of these fluids (*ca.* 4 g./100 ml.) which, as Johnston (1955*a*) has shown, causes irregularity of the $1/S-c$ plot; this error is unlikely to exceed 10% of S_0 and cannot account for the whole of the differences between different samples.

There appear again to be marked differences of properties between ox, sheep and human fluids, while the latter show considerable variations amongst themselves.

It is noticeable that, amongst the group of human fluids, the estimated values of d(1/*S*)/dc and of $[\eta]_c$ do not run parallel. It is therefore likely that errors of concentration do not contribute the whole variation of either of these quantities, and that there are some genuine differences from sample to sample. This confirms the conclusion drawn from Fig. 1 that different hyaluronic acids differ in their specific properties. Our values of S_0 and d(1/*S*)/dc for human fluids fall within the same range as those reported by Johnston (1955*b*).

Particle dimensions. Ogston (1953) has shown that indications of particle dimensions can be obtained from the measured values of S_0, d(1/*S*)/dc and $[\eta]_c$. Such values must in any case be accepted with caution and this should be increased, in the present case, by the relative uncertainty of the experimentally determined quantities for human and sheep fluids. Nevertheless, a comparison of the particle dimensions of hyaluronic acids from

different sources is probably valid. The striking differences between the values of the (unsolvated) molecular weight M, shown in Table 2, are certainly real. The large values of the hydrodynamic specific volume V' are consistent with the random-coil form of particle, deduced for ox hyaluronic acid by Ogston & Stanier (1951) and, as would be expected, V' varies in parallel with M. Values of the ellipticity J are in some cases rather large for a simple random-coil, but it is doubtful whether any conclusion can be drawn from this. It should be noted that the values of V' and J given in Table 2 for ox hyaluronic acid differ from those of Ogston & Stanier (1952) because of the new choice of constants noted by Ogston (1953).

The reality of the differences of M are shown by the fact that considerable amounts of hyaluronic acid present in a human traumatic fluid passed through a glass filter which entirely prevented the passage of ox hyaluronic acid.

SUMMARY

1. Viscosity measurements have been made on a small series of traumatic and pathological synovial fluids from human knee joints; also on three samples from ankle joints of normal sheep.

2. Sedimentation measurements have been made on a few of these fluids.

3. Comparison of variation of viscosity with velocity gradient, with its value at zero velocity gradient, suggests the existence of inter-species and possibly intra-species differences in the specific properties of hyaluronic acid. Sedimentation measurements appear to confirm this and suggest that human hyaluronic acids have considerably lower particle weights ($1-4\times10^6$) than those of sheep ($5-6\times10^6$) or ox (10^7).

4. Specific properties of human synovial fluid hyaluronic acid cannot, at present, be satisfactorily compared on a basis of concentration estimated from weight of mucin.

We are much indebted to Mr R. G. Taylor of the Wingfield-Morris Hospital, and to the Director and Staff of the Rheumatism Research Unit, Canadian War Memorial Hospital, Taplow, for samples of human synovial fluids; to the Director of the Nuffield Institute for samples of sheep synovial fluids; and to the Oxford Co-operative Society for facilities at their slaughterhouse. This work was assisted by a research grant from the Medical Research Council.

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Metabolism of β-Mercaptoethylamine

1. IN MICE

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(*Received* 29 *June* 1954)

Bacq *et al.* (1953) have shown that 3 mg. of β-mercaptoethylamine injected immediately before irradiation protect mice against 700 r. of X-rays. The percentage of ^{35}S remaining in the body of a mouse at various times after intraperitoneal injection of 3 mg. [^{35}S]β-mercaptoethylamine has previously been determined (Verly, Bacq, Rayet & Urbain, 1954), but for the understanding of the duration of the very short-lived radioprotection, it is more interesting to measure the ^{35}S that is still present in β-mercaptoethylamine (cysteamine) or β:β′-diaminodiethyldisulphide (cystamine) molecules. This paper presents the results of such measurements.

* Associé du Fonds National Belge de la Recherche Scientifique.

EXPERIMENTAL

Synthesis of [^{35}S]β-*mercaptoethylamine hydrochloride*

This compound was prepared as follows (see also Mills & Bogert, 1940; Bestian, 1950). $Ba^{35}S$ was ground in a mortar with some water, then transferred to an H_2S generator where it was made to react with an excess of phosphoric acid.

The $H_2{}^{35}S$ liberated was dissolved in methanol cooled to −78° and ethyleneimine in methanol at −78° slowly added to the stirred solution containing an excess of $H_2{}^{35}S$. Stirring was continued while the solution was allowed to come to room temp., when the excess $H_2{}^{35}S$, then the methanol, were distilled off.

The residue was washed several times with anhydrous ether, then dried *in vacuo*. The —SH groups were titrated with I_2 giving a result equal to 100·5% of the theoretical for mercaptoethylamine.

The substance was dissolved in water and titrated with HCl to pH 6·5–7; the quantity of HCl was the theoretical required to bring an equal amount of mercaptoethylamine to the same pH (i.e. to neutralize 97·5% of the base).

Ampoules were then filled with the solution and sterilized but titration of —SH groups with iodine, after sterilization, gave only 85–88% of the theoretical for mercaptoethylamine.

Chromatograms of the sterilized solution, after complete oxidation with I_2, on Whatman no. 1 paper, developed with N-HCl-saturated butanol, or with a water-saturated mixture of collidine–lutidine (1:1, by vol.), showed, in every instance, only one radioactive spot, the R_F of which was that of cystamine dihydrochloride chromatographed under the same conditions.

It appears therefore that the sterilized solution contains exclusively cysteamine (85–88%) and cystamine (12–15%) hydrochlorides. It was used for intraperitoneal injection into mice.

Isolation of cysteamine–cystamine from tissues

At suitable times after injection the mice were killed by a blow on the neck and immediately put in dry ice. The frozen animals were pulverized in a meat grinder. The ground tissues from one mouse were added to 200 ml. of water containing an accurately weighed amount of cystamine dihydrochloride (about 300 mg.), and the suspension was quickly brought to the boiling point and kept boiling for 1 hr.

The cooled suspension was filtered through cotton wool, the filtrate acidified with HCl, an excess of I_2 added to oxidize any thiol to disulphide and the solution extracted with diethyl ether. The aqueous phase was filtered through a 25 ml. Amberlite IR-120(H) column and the resin washed with 200 ml. water; filtrate and washings, which contained the acid metabolites were discarded. The substances with basic groupings adsorbed on the ion-exchanger were eluted with 500 ml. of 3N-HCl.

The acid eluate was evaporated to dryness under reduced pressure. The residue, dissolved in water, was filtered through a 25 ml. Amberlite IRA-410(OH) column and the resin washed with 100 ml. of water. This filtrate contained the cystamine while ampholytes remained on the column.

The basic solution was evaporated to about 5 ml. under reduced pressure so that volatile bases distilled. HCl was then added and the now-acid solution evaporated to dryness.

The residue was extracted with ethanol; sparingly soluble mineral salts were removed by filtration and the filtrate was evaporated to dryness.

This final residue was taken up in 4–6 ml. of methanol, the solution filtered and cystamine dihydrochloride was precipitated by addition of 12 ml. of acetone. The precipitate stood a few hours in the refrigerator, then was centrifuged and the supernatant decanted; the precipitate was washed with acetone and dried.

The precipitate was twice crystallized in the following way: the solid was dissolved in a mixture of ethanol–water–conc. HCl (12:1:1, by vol.) (Eldjarn, 1954) with slight warming, the solution was filtered, and diethyl ether was added to the filtrate so that the precipitate that formed just disappeared on boiling. The crystals were allowed to form at 20°, then in the refrigerator, and were washed with ether, then dried *in vacuo* at 78°.

The m.p.'s of the cystamine dihydrochloride samples thus isolated are recorded in Table 1. During recrystallizations the specific activity increased slightly. Chromatograms of samples 1 and 2, on Whatman no. 1 paper, developed with a water-saturated mixture of collidine–lutidine (1:1, by vol.), showed, in each case, one radioactive spot the R_F of which was that of cystamine dihydrochloride chromatographed under the same conditions.

Determinations of radioactivity

A portion of the ^{35}S cysteamine hydrochloride solution was oxidized with Benedict's reagent and the sulphate formed was precipitated as benzidine sulphate (Fiske, 1921). Cystamine dihydrochloride was oxidized with HNO_3 in sealed tubes (Carius) and the resultant sulphate turned into benzidine sulphate (Niederl, Baum, McCoy & Kuck, 1940).

Measurements of radioactivity were performed on the benzidine sulphate precipitates using the Tracerlab windowless flow counter in the Geiger region. Results were corrected for background, selfabsorption and radioactive decay and are expressed in counts/min./total sample.

RESULTS

The sterilized solution of [^{35}S]β-mercaptoethylamine hydrochloride was found to be 0·0824M and 0·5 ml. ($5·37 \times 10^6$ counts/min.) was injected intraperitoneally into each of seven mice (C 57 black) which weighed 20 g.

Table 1. *Properties of the isolated cystamine dihydrochloride*

For details see text.

Time after injection (hr. min.)	0·15		1·00	6·00		24·00	
Mouse no.	1	2	3	4	5	6	7
Total radioactivities (counts/min. $\times 10^{-6}$)	3·87	3·96	2·00	0·48	0·30	0·11	0·09
Total activities expressed as % of injected activity	72	74	37	9	6	2	2
M.p.'s ° (corr.)	217·5–218·5	218–219	218·5–219	218–219	217·5–218·5	218–219	218·5–219

Two mice were killed after 15 min., one after 1 hr., two after 6 hr. and two after 24 hr. The total [^{35}S]β-mercaptoethylamine and β:β'-diaminodiethyldisulphide of each animal was measured with results recorded in Table 1.

DISCUSSION

The experimental procedure used for cysteamine–cystamine isolation has been devised to measure 'free' [^{35}S]cysteamine–cystamine. It is not known whether part of the 'bound' cysteamine (pantetheine, coenzyme A) may not also be involved, but this is not important if 'free' cysteamine cannot be bound and thus introduce radioactivity into such molecules as coenzyme A. It seems that such is actually the case, since the S of cysteamine is not utilized for cystine biosynthesis, and cysteamine does not seem to be directly used for pantetheine and coenzyme A biosyntheses (Brown & Snell, 1953; Pierpoint & Hughes, 1954).

Total ^{35}S (Verly *et al.* 1954) and [^{35}S]cysteamine–cystamine remaining in the mouse body after intraperitoneal injection of the same amount of labelled cysteamine are compared in Fig. 1, in which activities are expressed as percentages of the injected dose. Attention must be drawn to two facts: in the previous work (Verly *et al.* 1954) cysteamine benzoate was used in place of cysteamine hydrochloride, but it is unlikely that the anion has any influence on mercaptoethylammonium ion metabolism; also the total ^{35}S determinations by the Na_2O_2 method (Verly *et al.* 1954) on the whole mouse gave results which are 5–10 % low.

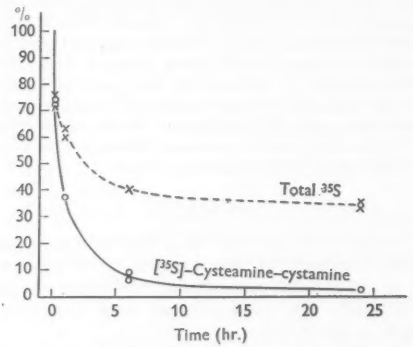

Fig. 1. Total ^{35}S (from Verly *et al.* 1954) and 'free' [^{35}S]-cysteamine–cystamine in the mouse body after injection of about 3 mg. of labelled cysteamine. The activities are expressed as percentage of the injected dose.

The curves of Fig. 1 show that 15 min. after the injection, most of the ^{35}S still in the organism is present as 'free' cysteamine–cystamine; after 24 hr. 'free' [^{35}S]cysteamine–cystamine is negligible although 34 % of the injected ^{35}S is still in the body of the mouse.

These facts could be explained if during the first minutes [^{35}S]cysteamine is excreted unchanged in the urine, but that later on the loss of labelled cysteamine is also dependent on tissue metabolism and the kidneys excrete a mixture of [^{35}S]cysteamine–cystamine and labelled metabolites, the latter become more and more predominant as time goes on.

SUMMARY

1. A method is described for the synthesis of [^{35}S]cysteamine–cystamine and for its determination in a whole mouse.

2. It was found that 50 % of 3 mg. of intraperitoneally injected labelled cysteamine disappear from the body of a mouse after about 40 min. After 24 hr., only 2 % are left.

The expenses of this work have been defrayed by grants from the Institut Interuniversitaire des Sciences Nucléaires and the Conseil Supérieur de la Sécurité Civile.

The authors wish to express their thanks to G. Espreux and A. Silvestre for their able assistance.

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Metabolism of β-Mercaptoethylamine

2. IN THE DOG

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(*Received* 29 *June* 1954)

β-Mercaptoethylamine forms a part of coenzyme A but is not known to occur naturally in the free state, for instance as a hydrolysis product from coenzyme A. Since β-mercaptoethylamine affords good protection against irradiation by X-rays (Bacq *et al.* 1953), it seemed worthwhile to look for sulphur compounds which can be derived metabolically from β-mercaptoethylamine.

The persistance of ^{35}S in the body of the mouse after intraperitoneal injection of 3 mg. of [^{35}S]β-mercaptoethylamine has been studied (Verly, Bacq, Rayet & Urbain, 1954), and it has been shown that 1 hr. or more after the injection, only part of the ^{35}S still present in the organism is in β-mercaptoethylamine (cysteamine)–β:β′-diaminodiethyldisulphide (cystamine) molecules (Verly, Gregoire, Rayet & Urbain, 1954). It was concluded that the injected cysteamine was partly excreted unchanged in the urine and partly metabolized in the tissues, the metabolites being eventually excreted in the urine.

In this work, some labelled molecules that appear in the urine after injection of [^{35}S]β-mercaptoethylamine to a dog, have been identified and their amount measured.

EXPERIMENTAL

The synthesis of the [^{35}S]β-mercaptoethylamine hydrochloride has been previously described. The solution injected into the dog contained 85–88% of cysteamine and 12–15% of cystamine (Verly, Gregoire, Rayet & Urbain, 1954).

The total sulphur and the total sulphates were determined by the methods of Fiske (1921).

Isolation of taurine and of cysteamine–cystamine

To 25 ml. of the diluted urine sample, accurately weighed amounts (about 200 mg.) of taurine and cystamine dihydrochloride, about 40 mg. of Na_2SO_4 and 30 ml. of conc. HCl were added. The solution was allowed to boil 1 hr. After cooling, an excess of I_2 was added, followed by $BaCl_2$ to precipitate the sulphates. The solution was filtered and evaporated under reduced pressure.

The residue was taken up in water, the solution filtered and passed through a column of 25 ml. of Amberlite IR-120(H); the resin was washed with 200 ml. of water; the filtrate and washings (Fraction *A*) contained the taurine (Carsten, 1952). The substances adsorbed on the sulphonated resin were removed with 500 ml. of 3N-HCl; this eluate (Fraction *B*) contained the cystamine; it was worked up as previously described (Verly, Gregoire, Rayet & Urbain, 1954).

Fraction *A* was evaporated to dryness under reduced pressure. The residue was taken up in 2–3 ml. of water and the solution filtered; crystals appeared on addition of 25 ml. of ethanol to the filtrate. After standing 2 hr. in the ice box, the mother liquors were removed and the crystals washed with ethanol.

The taurine was twice recrystallized from a water–ethanol mixture (except samples 1*a*, 2*a* and 2*b* which were recrystallized three times); the crystals were dried at 78° *in vacuo*. The nitrogen content of the samples was determined by Kjeldahl's method; the results are recorded in Table 1. The last recrystallization did not alter the specific activity of the taurine samples.

The isolated cystamine dihydrochloride samples were twice recrystallized from an ethanol–water–conc. HCl (12:1:1, by vol.) and ether mixture. The m.p.'s of these samples are recorded in Table 1. The last crystallization did not change the specific activity of the cystamine dihydrochloride.

Determinations of radioactivity

A portion of the [^{35}S]cysteamine hydrochloride solution was oxidized with Benedict's reagent (Fiske, 1921). The crystalline taurine and cystamine dihydrochloride samples were oxidized with HNO_3 in sealed tubes (Carius) (Niederl, Baum, McCoy & Kuck, 1940). In every instance, benzidine sulphate precipitates were prepared. Radioactivity measurements were performed on these precipitates using the Tracerlab windowless flow counter in the Geiger region. Results were corrected for background, self absorption and radioactive decay; they are expressed in counts/min./total sample.

RESULTS

A 7 kg. male dog, anaesthetized with α-chloralose was injected, into the femoral vein with 153 mg. [^{35}S]β-mercaptoethylamine hydrochloride (104 mg. of the base) containing a radioactivity of $10{\cdot}75 \times 10^7$ counts/min. The ureters were catheterized and urine collected for 8 hr.; the receiver was changed every 2 hr.

Each 2 hr. specimen was filtered, then diluted to 100 ml. from which samples were used for determinations of total ^{35}S, free and esterified

* Associé du Fonds National Belge de la Recherche Scientifique.

Table 1. *Properties of the isolated taurine and cystamine dihydrochloride*

For details see text.

Sample		0–2 hr. (1)	2–4 hr. (2)	4–6 hr. (3)	6–8 hr. (4)
Taurine N content (%)	*a*	11·25	11·1	11·1	11·1
(theoretical value (11·19%)	*b*	—	11·1	10·9	11·0
Cystamine, 2HCl,	*a*	217–218·5	—	219–220	—
m.p.'s ° (corr.)	*b*	218–220	219–220	—	218–220

Table 2. *Radioactivities of materials isolated from dog urine after injection of* [^{35}S]*cysteamine*

	Total radioactivity (counts/min. × 10^{-5})			
Sample	0–2 hr.	2–4 hr.	4–6 hr.	6–8 hr.
Total ^{35}S	72·0	42·8	33·6	18·8
Total sulphates	18·5	20·9	17·7	11·7
Taurine	0·6	2·2	2·7	2·2
Cystamine, 2HCl	33·0	7·1	4·2	1·5

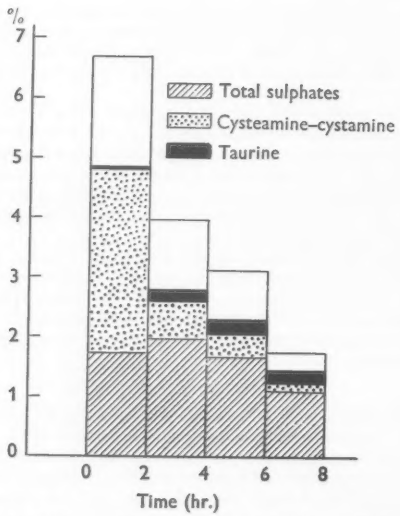

Fig. 1. Urinary excretion of total ^{35}S after intravenous injection of 104 mg. labelled cysteamine into a 7 kg. dog; radioactivity distribution between sulphates, taurine, cysteamine–cystamine and unidentified metabolites as percentages of the injected dose.

[^{35}S]sulphates, [^{35}S]taurine and [^{35}S]cysteamine–cystamine. Nearly all determinations were done in duplicate; the mean results are recorded in Table 2; Fig. 1 gives these results expressed as percentages of the injected dose. Similar results were obtained with another dog.

DISCUSSION

To calculate the activities of urine taurine and cysteamine–cystamine, the amounts of inactive taurine and cystamine added as carriers were assumed to be large compared with the amounts of labelled taurine and cysteamine–cystamine present in the very small volume (fourth of a 2-hour specimen) of urine used for analysis.

In 8 hr., 16% of the injected ^{35}S were excreted in the urine, 4% as cysteamine–cystamine. Since radioactive cysteamine–cystamine was mainly excreted during the first 2 hr. and practically none was present in the last urine sample collected, it may be concluded that from the 104 mg. of cysteamine injected to this 7 kg. dog, only 4–5% were excreted unchanged in the urine.

Fischer & Goutier Pirotte (1954) injected 150 and 190 mg. of cysteamine in the marginal vein of the ears of two rabbits and detected polarographically in the urine substances that discharged at the same potentials as cysteamine and cystamine. Expressed in cysteamine equivalents the amount of these substances excreted in the urine represented 30–35% of the injected dose.

An important fraction of the urinary ^{35}S is present as sulphate; there is also some excretion of labelled taurine. Eldjarn (1954) also reported the formation of taurine and sulphate from cystamine in the rat, rabbit and man.

It is clear from Table 2 and Fig. 1 that cysteamine–cystamine, taurine and sulphates do not account for the total ^{35}S excreted in the urine; there are thus other labelled metabolites still unidentified.

SUMMARY

1. A 7 kg. male dog was injected intravenously with 104 mg. of [^{35}S]cysteamine.

2. After 8 hr., 16% of the ^{35}S had been excreted in the urine. The greater part of it was present as sulphate, cysteamine–cystamine and taurine.

The expenses of this work have been defrayed by grants from the Institut Interuniversitaire des Sciences Nucléaires and the Conseil Supérieur de la Sécurité Civile.

The authors wish to thank Dr P. Fischer for injecting the radioactive compound into the dog and collecting the urine, and G. Espreux and A. Tremsky for their careful assistance.

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Sulphate Formation from Dimethylthetin in Rat Liver

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(*Received* 9 *July* 1954)

Dimethylthetin chloride, i.e.

$$\{(CH_3)_2.S^+.CH_2.CO_2H\}\ Cl^-,$$

has been established as a biological methyl donor on the basis of its ability to enhance the growth rates of rats fed on a methyl-free diet supplemented with homocystine (du Vigneaud, Moyer & Chandler, 1948; Maw & du Vigneaud, 1948) and its ability to promote methionine synthesis from homocysteine in the presence of liver preparations (Dubnoff & Borsook, 1948). Transfer of one methyl group from the thetin to homocysteine or homocystine takes place and the compound is believed to be converted into methylthioacetic acid. The methyl group of methylthioacetic acid, however, does not appear to be labile, since the acid does not have the growth-promoting properties of a methyl donor, and since the administration of trideuteriomethylthioacetic acid to rats does not result in the appearance of deuteriomethyl-labelled choline or creatine in the tissues (Maw & du Vigneaud, 1948). Furthermore, the acid is unable to methylate homocysteine *in vitro* (Dubnoff & Borsook, 1948).

In the intact rat the sulphur of dimethylthetin chloride, administered orally or by injection, has been shown to be converted to a considerable extent into urinary inorganic sulphate (Maw, 1953). This oxidation is considered to be connected with the methyl group lability, since ethylmethylthetin chloride and dimethyl-β-propiothetin chloride, i.e.

$$\{(CH_3)_2.S^+.CH_2.CH_2.CO_2H\}\ Cl^-,$$

which are also methyl donors, are likewise oxidized to sulphate, whereas diethylthetin chloride and trimethylsulphonium chloride, i.e. $\{(CH_3)_3.S^+\}\ Cl^-$, which are not methyl donors, are not so oxidized.

The present communication describes a series of *in vitro* experiments in which the formation of inorganic sulphate by rat-tissue preparations incubated with dimethylthetin chloride and some related compounds has been followed. It has been found that in contrast to its behaviour in the whole animal, dimethylthetin chloride alone is not oxidized to sulphate by liver or kidney slices, but that a mixture of the thetin and homocysteine gives rise to a higher output of sulphate in liver-slice preparations than does homocysteine alone. This implies that the oxidation of the thetin is dependent on its preliminary demethylation, as suggested by earlier animal experiments (Maw, 1953). Methylthioacetic acid, the most likely demethylation product, is readily oxidized by liver slices and to a lesser extent by kidney slices. Conversion of this compound into sulphate in the presence of fortified liver suspensions and acetone-dried liver powders has not so far been obtained. Its oxidation by liver slices has been compared with that of a number of related compounds, and also with that of cysteine, *S*-methylcysteine and methionine.

MATERIALS AND METHODS

Preparation of compounds

Dimethylthetin chloride and the sodium salts of mercaptoacetic (thioglycollic), methylthioacetic (*S*-methylthioglycollic) and ethylthioacetic (*S*-ethylthioglycollic) acids were prepared as described in a previous paper (Maw, 1953). Dithiodiglycollic acid, i.e. $(—S.CH_2.CO_2H)_2$, was prepared by the method of Westerman & Rose (1927) and converted into the neutral sodium salt. Sodium *S*-phenylthioacetate (*S*-phenylthioglycollate) was made from thiophenol and ethyl chloroacetate by a method similar to that used for the two alkylthioacetates. *S*-Methyl-L-cysteine was prepared by the reduction and subsequent methylation of L-cystine in liquid ammonia (du Vigneaud, Loring & Craft, 1934). L-Cysteine hydrochloride, DL-homocysteine, L-methionine and adenosine 5′-phosphate (AMP) were obtained from Roche Products Ltd., diphosphopyridine nucleotide (DPN) and cytochrome *c* from L. Light and Co. Ltd.

Procedure in metabolism experiments

Male black and white rats (wt. 120–150 g.) were killed by a blow on the head, the livers removed and slices cut in the usual way. Slices of approximately 200 mg. wet wt. were placed in 50 ml. Pyrex beakers containing 8 ml. of liquid medium, the composition of which is given below. The beakers were placed in an apparatus similar to that designed by Dubnoff (1948). This consisted of a rectangular wire tray fitted with rows of spring clips for holding the beakers, the tray being suspended in a thermostatically controlled water bath. The level in the bath was so adjusted that the beakers were immersed to a depth of 3·5 cm. (the depth of liquid within the beakers was 0·8 cm.). The beakers were totally enclosed by an airtight Perspex hood resting on the bottom of the tray and fitted with an inlet tube connected to a cylinder of the appropriate gas mixture, and an outlet tube. Beakers of volume 50 ml. were employed as reaction vessels because they allowed of a relatively large ratio of surface area to volume of the contents and thus of good contact with the gas phase within the hood. The tray was shaken mechanically in the bath at the rate of 100 oscillations/min. with an amplitude of 2·3 cm. The temperature of the bath was $37{\cdot}0 \pm 0{\cdot}1°$.

The medium in each beaker consisted of 5 ml. of either 'phosphate saline' or 'bicarbonate saline' (Krebs & Henseleit, 1932) in which $MgSO_4$ was replaced by $MgCl_2$ (Pirie, 1934*a*), together with 3 ml. of water containing the various compounds under investigation and any added reagents, all previously adjusted to pH 7·4. Metabolism experiments were of 2 hr. duration unless otherwise stated. During the first 30 min. the appropriate gas mixture (100% O_2 or N_2 in the case of the phosphate saline medium, and 95% O_2–5% CO_2 with the bicarbonate medium) after being saturated with water in a bubbler, was passed rapidly through the hood, the flow being reduced during the remaining 90 min. At the end of the period, the tissue slices were removed with forceps, washed with water, dried at 104° for 3 hr. and weighed. The contents of each beaker were deproteinized by the addition of 2 ml. of 20% trichloroacetic acid (TCA), and after standing for 30 min. filtered through a Whatman no. 1 paper.

Suspensions of fresh liver were prepared with the apparatus of Potter & Elvehjem (1936). The liver (2 g.) was ground up with a little ice-cold saline, made up to 50 ml. with more cold saline and filtered through two layers of muslin, 5 ml. of this suspension being used in each beaker in metabolism experiments, together with 3 ml. of water containing the added compounds. Acetone-dried liver powders were prepared in the usual way. Suspensions were prepared from 400 mg. of the powder made up to 50 ml. with saline and 5 ml. used in each beaker.

Tissue from one liver only was employed in each experiment. Beakers containing the tissue controls and the compounds under test were always prepared in duplicate, and the mean of each pair of resulting sulphate values taken.

Estimation of inorganic sulphate

The method used was that of Cuthbertson & Tompsett (1931) with certain minor modifications. A 0·5% solution of benzidine in acetone (5 ml.) was pipetted into finely tapered 15 ml. centrifuge tubes followed by 2 ml. of the TCA filtrates, the contents of each tube were mixed with a fine glass rod, and the tubes then placed in crushed ice in a stoppered thermos jar. A period of 1 hr. in ice has been suggested by Cope (1931) and by Medes & Floyd (1942) as being adequate for quantitative precipitation of benzidine sulphate under these conditions. Pirie (1934*a*) suggested a minimum period of 2 hr. but in the present experiments at least 4 hr. was considered necessary, and it was found more convenient to leave the tubes in ice overnight (18–19 hr.). The sides of each tube were then washed down with 0·5 ml. of ice-cold acetone and the tubes centrifuged for 10 min. at 1250 *g* at 2–4°. Centrifuging at room temperature led to variable analytical results. Dodgson & Spencer (1953) in their experiments experienced difficulty in removing the supernatant liquid without disturbing the precipitate. This was not encountered with filtrates obtained from the metabolism experiments described here, although occasionally it did happen when the method was being tested on pure solutions of sodium or potassium sulphate. The tubes were drained by inverting them on a hard filter paper and the precipitates resuspended in 2 ml. of ice-cold acetone and recentrifuged under the same conditions for 5 min. The tubes were again drained of supernatant liquid and the washing of the precipitates in acetone and the centrifuging repeated. To each tube, drained finally of acetone, was added 1 ml. of N-HCl; the tubes were placed in a boiling-water bath for 2 min. and the contents then washed out into 25 ml. graduated flasks with about 15 ml. of water.

The benzidine sulphate was estimated colorimetrically by the addition to each flask of 1 ml. of 0·1% $NaNO_2$ followed 1 min. later by 2 ml. of 1% thymol in 20% KOH. The volumes were made up to 25 ml. after 10 min. and the colour intensities compared after a further 10 min. against a water blank in a Hilger Spekker absorptiometer using Ilford 603 filters (max. transmission, 470–520 mμ.). Sulphate-S values were read from a calibration curve prepared from readings obtained with solutions of potassium sulphate containing the same amount of TCA as did the samples from metabolism experiments.

The above procedure gave very consistent results and the original calibration curve was checked frequently over a period of a year without showing any change. The reagents employed in the estimation gave rise to a small blank reading, constant for each set of reagents and filter papers used, and ranging from 4 to 10 μg. throughout the experimental series. The appropriate blank has been subtracted from all sulphate-S values recorded. It was determined by making up 5 ml. of the saline together with 2 ml. of 20% TCA to 10 ml., filtering the solution through a Whatman no. 1 paper and using 2 ml. of the filtrate in the above estimation. This procedure was carried out whenever a new batch of saline or other reagents was prepared, or whenever a new box of filter papers was opened.

All glassware was cleaned with nitric acid–chromic acid mixture and all solutions prepared with de-ionized water.

RESULTS

Inorganic sulphate in incubated liver slices

Relatively little data is available in the literature on the oxidative conversion of sulphur-containing compounds into sulphate by tissue slices and extracts. Pirie (1934*a*, *b*) observed the oxidation to sulphate of cysteine, cystine, glutathione, methionine and a number of other compounds by slices of

rat tissues, of which liver and kidney were the most active. Medes (1939) and Medes & Floyd (1942) confirmed these findings and extended their experiments to liver breis and extracts. Sulphate was also produced by tissue slices and extracts without the addition of sulphur-containing compounds to the medium. Medes (1939) gave average values for these tissue sulphate 'blanks' corresponding to 28·7 μg. sulphate-S/100 mg. dry wt. of tissue for liver slices and 54·1 μg. for kidney slices, both incubated for 2 hr. at 38° in a bicarbonate-containing medium.

In the present series of experiments sulphate-S determinations, carried out on liver slices incubated alone for 2 hr. at 37° in either phosphate saline or bicarbonate saline, gave values ranging from 14·0 to 35·4 μg. sulphate-S/100 mg. dry wt. of tissue (average of thirty-nine values, 24·8 μg.). Duplicate estimations in any one experiment generally agreed to within 5 μg. There was no noticeable difference between blank values obtained with either saline medium, and addition of 10 mg. of glucose to the medium had no effect on the amount of sulphate accumulating. Similar values were obtained with kidney slices (average 33·2 μg. of sulphate-S/100 mg. dry wt. of tissue/2 hr.). Sulphate estimations carried out on liver slices which were allowed to stand in either medium for 10 min. at room temperature gave values approximately half those for incubated slices. The sulphate appearing at room temperature may have arisen by diffusion from the slices during their short period in the medium, and it is probable, therefore, that the sulphate obtained after incubation was the result of further diffusion from the slices rather than of any oxidation occurring during the experiment.

Table 1. *Sulphate formation by rat-liver and -kidney slices in the presence of dimethylthetin chloride*

Contents of each beaker: 5 ml. saline, 3 ml. water containing 10 mg. dimethylthetin chloride neutralized to pH 7. Wet wt. of slices, approx. 200 mg. Incubation period, 2 hr. at 37°. Sulphate expressed as μg. S/100 mg. dry wt. of tissue.

Tissue	Type of saline used	Sulphate formed in the presence of: Control slice	Sulphate formed in the presence of: Dimethylthetin chloride
Liver	Bicarbonate	26·1	28·2
Liver	Bicarbonate	32·5	29·4
Liver	Bicarbonate*	21·3	23·3
Liver	Phosphate	26·3	24·1
Kidney	Bicarbonate	34·9	39·5

* Containing 10 mg. added glucose.

Experiments with dimethylthetin chloride

When dimethylthetin chloride was incubated with liver slices in either phosphate saline or bicarbonate saline, no increase in sulphate output was obtained (Table 1). This was an unexpected finding, in view of the extensive oxidation of the thetin which takes place in the whole animal. However, when DL-homocysteine was added to the medium, a larger amount of sulphate was formed than was obtained with homocysteine alone (Table 2). Homocysteine and homocystine are recognized methyl acceptors, both *in vivo* and *in vitro* and the data in Table 2 have been interpreted as indicating that dimethylthetin requires to be demethylated before being metabolized to sulphate. Glycocyamine, dimethylethanolamine and nicotinamide, known to be methyl acceptors specific for methionine, did not produce this sulphate increase in the presence of the thetin. The increase obtained with homocysteine although consistent was not large. This is probably accounted for by the fact that transmethylation reactions *in vitro* are relatively slow and, furthermore, those involving homocysteine are markedly inhibited by the presence of oxygen (Borsook & Dubnoff, 1947; Dubnoff & Borsook, 1948), an essential condition of the present experiments. In addition, the homocysteine was being lost from the system by oxidation to homocystine and sulphate and also by conversion into methionine by methyl donors

Table 2. *Sulphate formation by rat-liver slices in the presence of dimethylthetin chloride and* DL-*homocysteine*

Contents of each beaker: 5 ml. saline, 10 mg. dimethylthetin chloride in 2 ml. water, neutralized to pH 7, and/or DL-homocysteine in 1 ml. water, neutralized to pH 7, and water to 8 ml. Wet wt. of slices, approx. 200 mg. Incubation period, 2 hr. at 37°. Sulphate expressed as μg. S/100 mg. dry wt. of tissue.

Type of saline used	Amount of homocysteine present (mg.)	Sulphate formed in the presence of: Control slice	Dimethylthetin chloride	Homocysteine	Dimethylthetin chloride + homocysteine
Bicarbonate	5	27·1	26·5	108·7	139·6
Bicarbonate	5	31·2	—	73·8	123·7
Bicarbonate	10	22·9	23·8	113·7	174·6
Bicarbonate	10	—	—	141·3	174·9
Phosphate	10	23·8	—	86·5	124·6
Phosphate	10	27·0	—	84·2	110·4

Table 3. *Sulphate formation by rat-liver slices in the presence of sodium methylthioacetate and some related compounds*

Contents of each beaker: 5 ml. saline, 3 ml. water containing the compound under study (amount of each compound present equivalent to 64 μmoles, unless otherwise stated). Wet wt. of slices, approx. 200 mg. Period of incubation, 2 hr. at 37°. Sulphate expressed as μg. S/100 mg. dry wt. of tissue.

Compound under study	Amount in each beaker (mg.)	Type of saline used	Sulphate formed in the presence of: Control slice	Sulphate formed in the presence of: Added compound
Sodium methylthioacetate	8·2	Bicarbonate	20·7	135·6
	8·2	Bicarbonate	35·3	131·6
	8·0	Phosphate	14·9	82·8
	8·2	Phosphate	26·3	190·7
	6·0*	Phosphate†	19·5	16·2
Sodium mercaptoacetate	7·3	Bicarbonate	22·5	50·2
	7·3	Phosphate	18·1	54·5
Sodium ethylthioacetate	9·1	Bicarbonate	35·3	71·4
Sodium phenylthioacetate	12·2	Bicarbonate	35·3	31·2
Disodium dithiodiglycollate	7·2	Bicarbonate	22·5	68·8
	7·2	Phosphate	18·1	43·2
L-Cysteine hydrochloride	10·1	Bicarbonate	28·8	261·0
S-Methyl-L-cysteine	8·7	Bicarbonate	28·8	88·0
L-Methionine	9·5	Bicarbonate	33·7	58·8

* Equivalent to 47 μmoles.
† Under anaerobic conditions (100% N_2).

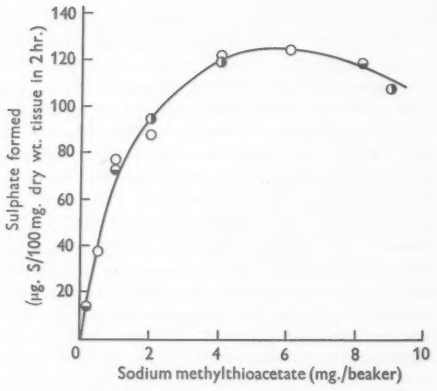

Fig. 1. Relationship between the amount of sulphate formed by rat-liver slices and the amount of sodium methylthioacetate present. The results shown were obtained from three separate experiments.

initially present in the tissue, as observed by Borsook & Dubnoff (1947). The sulphate increase reported could have arisen not only from the oxidation of the demethylation product of the thetin, but also from the oxidation of the methionine formed in equivalent amounts from homocysteine. Methionine is oxidized at a slower rate than is methylthioacetic acid, however (see Table 3), and would contribute only partially to the increase. In any case, whether the sulphate increase originated from methylthioacetic acid, from methionine, or from both compounds, it would still implicate methyl transfer as the initial metabolic step.

Oxidation of methylthioacetic acid and related compounds by liver slices

Methylthioacetic acid was oxidized aerobically, but not anaerobically, to sulphate by liver slices, and also to a slight extent aerobically by kidney slices. In Table 3 are shown figures for its oxidation by liver slices together with data for some related compounds including L-cysteine, *S*-methyl-L-cysteine and L-methionine, all compounds being studied at comparable concentrations. The acid was oxidized to about the same extent as DL-homocysteine (Table 2), less readily than L-cysteine and more so than *S*-methyl-L-cysteine or L-methionine. It also gave rise to sulphate to a greater extent than mercaptoacetic, dithiodiglycollic and ethylthioacetic acids, a similar situation to that observed in the whole animal (Maw, 1953). *S*-Phenylthioacetic acid was not oxidized to sulphate, and this has also been found to be the case in the whole rat (Maw, unpublished observations).

Fig. 1 shows the relationship between concentration of methylthioacetic acid (as the sodium salt) in the medium and the amount of sulphate formed, the latter being expressed as μg. S/2 hr., from which the tissue sulphate 'blanks' have been subtracted. The wet weights of the tissue slices used were approximately 200 mg. The largest amount of sulphate was obtained at a level of methylthioacetic acid (sodium

salt) of 4–6 mg., corresponding to about 4% oxidation. At lower levels the percentage oxidation was higher, 6% at a level of 2 mg. and 10% at a level of 1 mg. This is of the same order as that obtained with equivalent amounts of cysteine, both in the present experiments and in those of Pirie (1934*a*) and Medes (1939).

Experiments with liver suspensions and acetone-dried powders

Attempts made to study the conversion of methylthioacetic acid into sulphate in more detail with suspensions in saline of fresh liver and acetone-dried liver powders, ranging in concentration from 2·5% to 10%, have so far been unsuccessful. Liver so treated completely lost its ability to oxidize the compound. Furthermore, addition of the following reagents either singly, in groups or altogether, did not restore the activity: glucose (7×10^{-3}M), $MgCl_2$ (10^{-2}M), $MnCl_2$ (10^{-3}M), phosphate buffer, pH 7·4 ($1{\cdot}2 \times 10^{-3}$M, used with bicarbonate saline only), DPN ($3{\cdot}8 \times 10^{-4}$M), cytochrome *c* ($4{\cdot}8 \times 10^{-6}$M), AMP ($7{\cdot}2 \times 10^{-4}$M) and sodium fumarate ($5{\cdot}4 \times 10^{-3}$M), the figures in brackets referring to the final concentrations of the reagents in each beaker. The last two reagents were used in place of ATP as in the experiments of Borsook & Dubnoff (1947).

The ability of suspensions of fresh liver or acetone-dried liver powders to oxidize mercaptoacetic acid was very much diminished, and in some experiments lost. An unfortified 2·5% suspension containing the same amount of tissue as used in the liver-slice experiments, was still able to oxidize L-cysteine, although only at about one-third to one-half the rate obtained with slices. DL-Homocysteine also formed sulphate under these conditions, but at a third of the rate of slices, whereas L-methionine was not oxidized, even in the presence of added phosphate, $MgCl_2$, AMP, sodium fumarate and a methyl acceptor, such as nicotinamide or glycocyamine. *S*-Methyl-L-cysteine also was not oxidized.

DISCUSSION

Dubnoff & Borsook (1948) have shown that dimethylthetin chloride is demethylated by liver preparations under anaerobic conditions in the presence of homocysteine, one methyl group being lost. Dubnoff (1949) subsequently reported that the product was methylthioacetic acid, although no supporting experimental evidence was provided. Since dimethylthetin chloride is oxidized to sulphate in the whole rat, the present finding that no oxidation took place in liver slices, but that together with homocysteine it resulted in a greater formation of sulphate than from homocysteine alone, suggests that demethylation is the initial step in the oxidation. This finds a parallel in the whole rat, in which out of a number of sulphonium compounds administered orally, only those which behaved as methyl donors to homocystine were converted into sulphate (Maw, 1953). The apparently specific role of homocysteine in sulphate formation is in accord with the fact that it is the specific acceptor in methyl transfer reactions from glycine betaine and the thetins.

Liver suspensions were unable to oxidize methylthioacetic acid, in contrast to liver slices. It seems likely that the loss of activity in disintegrated tissue is due to dislocation of enzyme systems involved in the earlier stages of the breakdown, e.g. those concerned with the removal of the *S*-methyl group or with fission of the sulphur from the rest of the molecule. In the terminal stages of the oxidation the forms in which the sulphur is most likely to be released from organic combination are hydrogen sulphide or sulphur dioxide, and these are both oxidized by liver and other tissue preparations (Smythe, 1943; Heimberg, Fridovich & Handler, 1953). It is of interest that liver suspensions were also unable to oxidize methionine and *S*-methylcysteine, possibly for a similar reason.

A survey of the literature shows that oxidation to sulphate in the animal is restricted to a relatively small number of organic sulphur compounds. Thus, in addition to the sulphur-containing amino acids and some of their derivatives, only certain aliphatic thiols and related disulphides, certain aliphatic thioethers, generally containing the CH_3S— group, and a few miscellaneous sulphur compounds are oxidized in this way (Hill & Lewis, 1924; Westerman & Rose, 1928; Shen & Lewis, 1946; Fromageot, 1947). The compounds methylthioacetic acid and ethylthioacetic acid studied here, together with β-hydroxyethyl methyl sulphide (Maw, 1953) provide an addition to the group of thioethers which can give rise to sulphate, while the behaviour of *S*-phenylthioacetic acid in the whole rat and in rat-liver slices strengthens the original view of Hill & Lewis (1924) that aromatically bound sulphur is poorly oxidized, if at all. Sulphonium compounds which possess labile methyl groups must be considered as a further group of compounds capable of giving rise to sulphate. In the light of the present work it is suggested that there is no direct pathway for the oxidation of the sulphur of these compounds, but that oxidation only takes place after their conversion into thioethers by transmethylation. Their position in the general scheme of sulphur metabolism may therefore be compared with that of the sulphate-forming disulphides referred to above, which also require to undergo preliminary conversion to directly oxidizable compounds, in this case thiols.

SUMMARY

1. Dimethylthetin chloride incubated with rat-liver or -kidney slices in either bicarbonate or phosphate salines was not oxidized to sulphate, but the compound incubated with homocysteine gave rise to a larger amount of sulphate than was obtained with homocysteine alone.

2. Methylthioacetic acid was oxidized to sulphate by liver slices and to a lesser extent by kidney slices. Ethylthioacetic, mercaptoacetic and dithiodiglycollic acids were also oxidized by liver slices, whereas *S*-phenylthioacetic acid was not.

3. Suspensions of fresh liver or acetone-dried liver powders were unable to oxidize methylthioacetic acid. Addition of the following reagents singly, in groups, or altogether did not restore the oxidative ability of the tissue: $MgCl_2$, $MnCl_2$, glucose, diphosphopyridine nucleotide, cytochrome *c* and adenosine 5′-phosphate with sodium fumarate.

Grateful acknowledgement is made to the Endowment Fund of St Thomas's Hospital for aid in the purchase of materials and equipment.

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Studies on the Volatile Fatty Acids of Sheep Blood with Special Reference to Formic Acid

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(*Received* 14 *July* 1954)

The production of volatile fatty acids (VFA's) in the rumen, and their subsequent absorption is well established. The recognition of the nutritional significance of these acids to the ruminant (cf. Phillipson, 1948) has led to many investigations of the VFA's in the blood of these animals. Reid (1950) made a careful study of the uptake of VFA from the peripheral circulation by the tissues of the sheep, and VFA levels in the blood of cattle were reported by McClymont (1951*a*). In earlier work values were reported for the concentration of circulating VFA in men and dogs (McClendon, 1944), and in the sheep, pig, pony and rabbit (Barcroft, McAnally & Phillipson, 1944). Dog blood was subsequently investigated by Phillipson (1947).

Investigation of the nature of the blood VFA's, however, had to await the development of reliable methods of analysis, based on partition chromatography (Elsden, 1946; Moyle, Baldwin & Scarisbrick, 1948). Reid (1950), using chromatographic methods, found that about 90% of the VFA of sheep blood was acetic acid, and similar findings with respect to cattle blood were reported by McClymont (1951*a*). The VFA's of goat blood have recently been examined chromatographically by Craine & Hansen (1952).

In each of these chromatographic investigations of blood VFA's the acids were first distilled from mercuric oxide by the method of Friedemann (1938) to remove acidic substances which might interfere with the subsequent analysis. Formic acid is destroyed by this procedure. Furthermore, the

* This work was started while the author was seconded to the Rowett Research Institute, Bucksburn, Aberdeenshire.

liquid–gel partition methods used in these chromatographic analyses would not readily distinguish formic acid from acetic acid. The recently developed gas–liquid partition chromatographic method (James & Martin, 1952) separates these and the other lower VFA's, and the application of this method to the analysis of sheep blood during the course of another investigation (Annison & Pennington, 1954) gave results which suggested that formic acid comprised 10–30 % of the VFA's of the venous blood of sheep under normal conditions. The further investigation of this finding is the subject of the present paper.

The studies were extended to include an examination of the VFA content of urine, saliva, gastric juice and cerebrospinal fluid. The possibility that formate was produced by bacterial fermentation in some part of the alimentary tract and subsequently absorbed into the blood stream was also investigated.

MATERIALS AND METHODS

Experimental animals. Three Clun Forest sheep were fed once daily on rations of hay (1 kg./day), casein (1 kg. hay and 150 g. casein/day) and dried grass (400 g. dried grass and 300 g. hay/day) respectively. The rations were usually consumed within 2 hr. The sheep maintained on dried grass had a permanently exteriorized carotid artery. Additional animals kept for other purposes were available when required.

Sampling procedures. Venous blood was obtained by withdrawal from the jugular vein, and arterial samples were taken from the sheep with an exteriorized carotid artery. Heparin was used as an anticoagulant throughout the investigation.

Urine samples were obtained from ewes by inserting a polythene catheter into the bladder and allowing free flow under gravity into a collection vessel. The animals were restrained during the collection period.

Small quantities of saliva were obtained from sheep by washing the mouth with water and then applying gentle suction to a rubber tube held in the mouth of the animal. In addition, samples were obtained from sheep anaesthetized with Nembutal (sodium pentobarbitone). The mouth and teeth were washed and saliva was allowed to drain from the mouth which was held slightly open with retractors. The first 50 ml. of saliva was rejected.

Gastric juice was obtained from a 10-week-old lamb which had an innervated gastric pouch; this was prepared by Mr K. Hill for another purpose.

Cerebrospinal fluid was taken from sheep by suboccipital puncture under light anaesthesia (Nembutal).

Determination of VFA's in body fluids

Blood. Most published methods for determining total blood VFA's involve deproteinization of the blood and subsequent steam distillation of the strongly acid filtrate (McClendon, 1944; Phillipson, 1947). A suitable volume of distillate is collected and titrated with alkali under CO_2-free conditions and a second and equal volume of distillate is treated similarly to provide a correction figure (cf. Phillipson, 1947). In no case has the validity of this type of procedure been checked by subsequent VFA analysis of the distillates, since suitable methods for the small quantities involved were not available. The complete recovery of added VFA's from blood examined by these methods is a valuable check on possible losses associated with the deproteinization procedures but gives no information on the nature of the acidic substances estimated.

A method for determining total blood VFA levels has been developed which yields sufficient acid for subsequent chromatographic analysis by the procedure of James & Martin (1952). It was obviously desirable that the volume of blood used for each analysis should be as small as possible. The limiting factor was the amount of VFA required for chromatographic analysis. In order to scale down the method, the strength of the alkali in the recording burette (for details see James & Martin, 1952) was reduced from 0·04 to 0·01 N. However, the solutions of the sodium salts of the VFA's to be analysed were more dilute than those recommended by James & Martin (1952), and the extraction of VFA's from such solutions by the method of these authors gave low and irregular recoveries (50–70 %, w/w). These low recoveries were attributed to adsorption of VFA on the Na_2SO_4 used in the extraction procedure. Results were obtained indicating that, under standard conditions, the losses from this cause increased with the chain length of the VFA examined. Reduction of the quantity of anhydrous Na_2SO_4 employed gave increased recoveries and the procedure outlined below, which allows the analysis of mixtures containing 5–20 μmoles of VFA, was finally adopted.

Blood samples (20 ml.) were laked with 70 ml. water and deproteinized with 10 ml. of freshly prepared 25 % (w/v) metaphosphoric acid. After standing 20 min. to allow complete coagulation of the precipitated protein, the suspension was filtered at the pump. The flask and precipitate were washed with 25 ml. of water. The filtrate and washings were neutralized to pH 8·0–8·5 with 5 N-KOH and concentrated *in vacuo* to a volume of 3–4 ml. at 30–40° using a water pump and a combined magnetic stirrer–hotplate (Baird and Tatlock, London Ltd., Chadwell Heath, Essex). This solution was carefully washed into a 10 ml. volumetric flask, adjusted to pH 2·8–3·0 with 10 N-H_2SO_4 (bromophenol blue indicator) and made up to 10 ml. Duplicate samples (4 ml.) were distilled in a Markham still and two portions of distillate (100 ml. each) were collected and titrated with 0·01 N-NaOH under CO_2-free conditions using phenolphthalein as indicator. The titration of the second 100 ml. of distillate served as a blank. The distillates were concentrated and the sodium salts of the VFA's were dried in a small test tube (6 × 0·6 cm.) and retained for chromatographic analysis. In order to transfer the VFA's to the gas–liquid partition columns, 40 μl. of 30 % (w/v) aqueous H_3PO_4 were added from a micropipette to the sodium salts. After thorough mixing the solution was transferred to the top of a micro-column similar to that described by James & Martin (1952), but containing a smaller quantity of Na_2SO_4 and Celite. A 0·6 cm. column of anhydrous Na_2SO_4 (0·08–0·09 g.) above which was placed a 0·2–0·3 cm. column of Celite in a tube of 0·3 cm. internal diameter was employed. The test tube was rinsed with 0·4 ml. of VFA-free ether, which was transferred to the micro-column and passed through under slight pressure. This procedure was repeated with a further 0·3 ml. of ether, the same pipette being used throughout. The VFA's were transferred from the ethereal solution to the gas–liquid partition column as described by James & Martin (1952).

Improved separations of VFA's were obtained by using partition columns of modified composition. The amount of stearic acid in the liquid phase (DC 550 silicone, Albright and Wilson Ltd., Oldbury, Birmingham) was increased from 10 to 15% (w/w). Under these conditions 'tailing' was considerably reduced and good separations of formic acid and acetic acid were obtained. An operating temperature of 135° and a N_2 flow rate of 15 ml./min. were employed throughout. All percentages of VFA's are expressed on a molecular basis.

Several reagents for deproteinizing blood which had been laked with water were examined, including $ZnSO_4$–$Ba(OH)_2$ (Weichselbaum & Somogyi, 1941), $ZnSO_4$–NaOH (Somogyi, 1930), perchloric acid (Grauer, Mendl, Strauss & Neuberg, 1950) and metaphosphoric acid (Phillipson, 1947). The $ZnSO_4$-$Ba(OH)_2$ method is theoretically the most suitable, since the reagent is completely eliminated with the precipitated protein, but the recoveries from sheep blood of added VFA were always low (70–80%). Similar results were obtained with the Somogyi reagent (Somogyi, 1930), and the metaphosphoric acid method of Phillipson (1947) was found to be the most satisfactory. Recoveries from blood of added VFA were invariably in the range 95–100%.

The deproteinized blood filtrates were distilled at pH 2·8–3·0 to minimize the interfering effects of other substances normally present, such as pyruvic and lactic acids and chloride, which are partially steam volatile under acid conditions. Control experiments established that at pH 3·0 only 5–8% of pyruvic acid steam distilled under the conditions used for the blood filtrates, but at pH 1·5 about 30% steam distilled. Similarly, at pH 3·0 the influence of chloride, lactic acid and β-hydroxybutyric acid, when present at concentrations similar to those encountered in blood, is negligible. The utility of steam-distilling VFA's under conditions of minimum acidity when interfering substances are present was recognized by Scarisbrick (1952), who estimated blood VFA's by steam distillation at pH 4·0. At this pH, formic acid cannot be steam distilled quantitatively, but at pH 3·0 complete recoveries of all of the lower VFA's were obtained.

The reliability of the method described above for blood VFA analysis was checked by performing ten analyses on a large pooled sample of sheep blood (Table 1). Good agreement was obtained between the total VFA level found by titration and the VFA accounted for by subsequent chromatographic analysis of the distillates.

Urine. Total VFA levels were determined by steam distillation. Samples of urine (5 ml.) were adjusted to pH 2·8–3·0 (bromophenol blue indicator) by the addition of 1·5 ml. of phosphate buffer (saturated aq. KH_2PO_4 adjusted to pH 2·8–3·0 with 88% (w/w) H_3PO_4) and 2 g. of $MgSO_4$, $7H_2O$ were added before the samples were steam distilled in a Markham apparatus. The distillate (150 ml.) was collected, neutralized with alkali and concentrated to a small volume (4–5 ml.). The acids were then redistilled in the Markham apparatus at pH 3·0, and two 100 ml. portions of distillate were collected and titrated with 0·01 N-NaOH under CO_2-free conditions, the titre of the second portion serving as a correction figure. The distillates were concentrated and retained for chromatographic analysis. Double steam distillation was found necessary, since a considerable amount of acidic substance other than VFA steam distilled at pH 3·0 in the first distillation, but after the second distillation the titratable acidity was all accounted for as VFA by the subsequent chromatographic analysis.

Saliva. Samples of saliva (5 ml.) were adjusted to pH 3·0 with 5 N-H_2SO_4 saturated with KH_2PO_4 (bromophenol blue indicator) and steam distilled in the Markham apparatus after the addition of 2 g. $MgSO_4$, $7H_2O$. Two 100 ml. portions of distillate were collected and titrated with alkali as described for urine VFA analysis and retained for chromatographic analysis.

Gastric juice. Duplicate samples (5 ml.) were adjusted to pH 2·8–3·0 (bromophenol blue indicator) by the addition of M-K_2HPO_4 and steam distilled in the Markham still after the addition of 2 g. $MgSO_4$, $7H_2O$. Two 100 ml. portions of distillate were collected and titrated with alkali as described for urine VFA analysis and retained for chromatographic analysis.

Cerebrospinal fluid. Samples (5 ml.) were analysed for VFA's as described for saliva.

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) and the ammonia, which was steam distilled in the Markham apparatus, was trapped in the boric acid reagent of Conway & O'Malley (1942) and titrated with standard acid.

Table 1. *Results of ten VFA analyses on the same sample of sheep blood (venous)*

Sample	Total VFA (m-moles/l.)	Molecular percentages of VFA's		
		Formic	Acetic	Propionic
1	1·37	12	87	1
2	1·70	12	87	1
3	1·36	13	86	1
4	1·24	13	86	1
5	1·36	12	87	1
6	1·37	11	88	1
7	1·36	12	87	1
8	1·37	13	86	1
9	1·32	14	85	1
10	1·40	13	86	1

RESULTS

Formate in sheep blood

Identification of formic acid in sheep blood. Irrespective of the methods employed to isolate the VFA's from sheep blood, subsequent chromatographic analysis invariably gave rise to an acid zone on the gas–liquid chromatogram which, from its retention volume under the conditions employed, was provisionally identified as formic acid. The problem at this stage was twofold. It was necessary not only to confirm the identity of this acid, but also to establish whether it is a normal constituent of sheep blood, or whether it is produced subsequent to the withdrawal of the sample either as a result of standing or by the procedures employed to isolate the blood VFA's.

The latter point was investigated first and, in preliminary experiments, it was shown that the VFA content of a sample of venous blood did not change appreciably on standing for several hours at room temperature. To investigate whether or not

the formic acid observed in sheep blood was produced during the isolation procedures, a large sample of blood (200 ml.) was heparinized and examined for VFA's by the following methods. (*a*) Deproteinization with metaphosphoric acid (procedure described above). (*b*) Deproteinization with $ZnSO_4$–NaOH (Somogyi, 1930); the filtrate was concentrated *in vacuo* and steam distilled at pH 3·0 as in (*a*). (*c*) A portion of the blood (60 ml.) was lightly centrifuged to remove red cells and the heparinized plasma dialysed at 0° against successive changes of distilled water for 4 days. The diffusible VFA was obtained by steam distilling the concentrated solution at pH 2–3. (*d*) Plasma obtained from a further portion of blood (100 ml.) was dialysed against an equal volume of distilled water at 0° for 2 days. The diffusate was acidified and exhaustively extracted with ether previously shaken with alkali to remove any VFA contaminants. The ether was then extracted with a slight excess of alkali (0·1N-NaOH) and this aqueous solution was divided into two equal portions, one of which was acidified and steam distilled to measure the VFA content. The other portion was evaporated to dryness and, in spite of a large amount of contaminating solid, examined directly by gas–liquid chromatography. The VFA extraction procedure described earlier was somewhat modified: the solid material was dissolved in 100 μl. of 40 % (w/v) aq. H_3PO_4 and twice the normal amount of anhydrous sodium sulphate was used.

The results of these analytical procedures are shown in Table 2. The small proportions of formate observed in procedures (*c*) and (*d*) are probably due to incomplete recoveries of VFA, but the presence in blood of an acid which behaves like formic acid revealed by these mild methods of extraction suggests that the acid is normally present in sheep blood.

The VFA's from a large blood sample (200 ml.) were isolated and examined chromatographically and a zone tentatively identified from its retention volume as formic acid was observed. Addition of formic acid to the sample before analysis did not give rise to a new zone but only enlarged the original zone. Distillation of the VFA mixture from HgO by the method of Friedemann (1938), before chromatographic analysis, either greatly reduced or eliminated the apparent formic acid. A further portion of the VFA mixture was examined chromatographically and the apparent formic acid was collected by removing the titration cell contents after the emergence from the column of the first acid zone. This acid was isolated by steam distillation and examined chromatographically alone and with added formic acid. In each case only one acid zone was observed, with a retention volume characteristic of formic acid. In a further experiment the apparent formic acid was isolated from two samples of sheep blood by the procedure described above. These acid samples were examined for formic acid by the colorimetric method of Grant (1948), which is based on the reduction of the acid to formaldehyde and measurement of the colour produced with chromatotropic acid. The colours produced were similar to those given by formic acid standards examined at similar concentrations. The method of Grant (1948) is not specific for formic acid. In subsequent experiments the VFA's from four samples of sheep blood were isolated by the standard procedure described earlier and analysed for formic acid by this colorimetric method and by gas–liquid partition chromatography (Table 3). The poor agreement is attributed to the difficulty experienced in obtaining reproducible results by the chromotropic acid method of Grant (1948).

Conclusive proof of the identity of the apparent formic acid was obtained by the isolation of the acid from sheep blood.

Isolation of formic acid from sheep blood. Samples of venous blood (290 ml.) were withdrawn from two sheep 5 hr. after feeding and pooled. The blood was laked with 1 l. of water and deproteinized with 250 ml. of 25 % metaphosphoric acid. The filtrate was neutralized with 5N-KOH, concentrated *in vacuo* to about 100 ml. and steam distilled after acidifying to pH 2–3 with 10N-H_2SO_4. The distillate

Table 2. *Results of the chromatographic analysis of the VFA's of a sheep blood sample from which the VFA's were isolated by the methods indicated*

Method of isolation (see text)	Composition of VFA fraction (molecular proportions)		
	Formic	Acetic	Propionic
(*a*) Standard procedure	17	82	1
(*b*) Deproteinization of blood by $ZnSO_4$–$Ba(OH)_2$ technique	16	83	2
(*c*) Dialysis and steam distillation	14	84	2
(*d*) Dialysis and ether extraction	12	85	3

Table 3. *Results of analyses of blood samples for formic acid by the chromatographic and chromotropic acid methods*

Sample	Total VFA (m-moles/l.)	Formate content of isolated VFA's (moles/100 moles VFA)	
		Chromatographic analysis	Chromotropic acid method
1	0·9	12	10
2	1·0	18	13
3	0·8	15	12
4	0·7	21	20

was neutralized with alkali, concentrated to about 10 ml. and steam distilled from acid solution (pH 2–3) in the presence of added $MgSO_4$, $7H_2O$ (6 g.). The distillate (200 ml.) was titrated with 0·1 N-NaOH, which indicated that the total VFA present was 0·80 m-moles. Chromatographic analysis showed that the sample contained 28 % formic acid or 0·22 m-moles (10·1 mg.). This formic acid was isolated from the other VFA's by gas–liquid partition chromatography. The sample was divided into 10 approx. equal portions which were run separately on partition columns. After the emergence of the formic acid zone the cell contents were collected, and the remainder of the VFA's were quickly eluted from the column using a high rate of nitrogen flow. The formic acid from the pooled cell contents was recovered by steam distillation (0·19 m-moles). Chromatographic analysis of this sample showed that formate (94 %) and acetate (6 %) were present.

The identity of the acid was confirmed by the analysis of its barium salt which was precipitated from aqueous solution with five volumes of ethanol. This procedure was repeated once. Yield of barium salt, 23 mg.: chromatographic analysis indicated that formate (96 %) and acetate (4 %) were present. (Found: C, 10·6; H, 1·2. Calc. for mixture of barium formate, $Ba(O_2CH)_2$, (96 %) and barium acetate, $Ba(O_2C_2H_3)_2$, (4 %), C, 10·88; H, 0·94 %.) The elementary analyses were made by Drs Weiler and Strauss, Oxford.

Further confirmatory evidence of the identity of this acid was obtained by utilizing the formic hydrogenylase enzyme system of *Escherichia coli* (Woods, 1936). The isolated formate (sodium salt) was incubated at 37° with a suspension of *Esch. coli* (0·39 mg. of bacterial-N/ml.) in Warburg manometric flasks (one side bulb), with KOH in the centre wells and under a gas phase of N_2. The bacterial suspension (2 ml.) was placed in the main compartment and the isolated formate (14·8 μmoles) in the side arm. Control flasks containing 15 μmoles of authentic formate were examined in a similar manner. The volume of hydrogen liberated from the test samples was almost identical with that expected on the basis of the formate content.

VFA's of sheep blood

Daily variation in sheep blood VFA's. Blood samples for VFA analysis (20 ml.) were withdrawn at 2 hr. intervals throughout the course of a day from two sheep maintained on the hay and casein diets respectively. The results are shown graphically in Fig. 1. The increased level of blood VFA's after feeding is due almost entirely to a rise in the acetate content of the blood, the formate levels remaining fairly steady.

Blood VFA's of fasted sheep. Food was withheld from the two sheep maintained on diets of hay and casein respectively, and blood samples were withdrawn for VFA analysis at 4 hr. intervals throughout a period of 48 hr. The results shown in Fig. 2 indicate that, although the level of acetate in blood fell markedly, there was only a slight decrease in the formate concentration.

Higher VFA's in sheep blood. Small amounts of butyric acid (< 1 %) were frequently observed when sheep blood was analysed for VFA's and traces of the C_5 acids were occasionally seen. When larger amounts of blood were examined, traces of all the C_4 and C_5 VFA's which normally occur in the sheep's

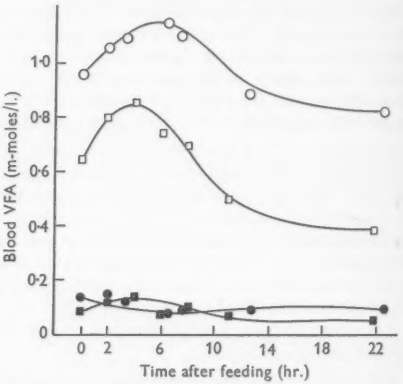

Fig. 1. Daily variation in blood VFA concentration (m-moles/l.) of sheep maintained on hay (total VFA's, □—□; formate, ■—■) and hay with added casein (total VFA, ○—○; formate, ●—●). Sheep fed during 0–2 hr. period.

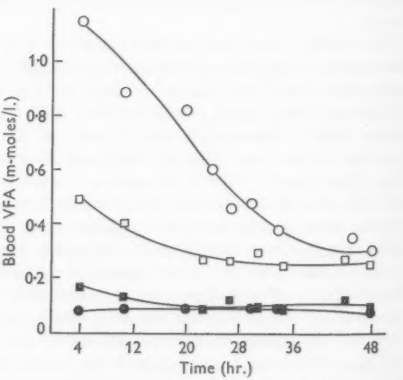

Fig. 2. Changes in blood VFA concentration (m-moles/l.) of fasted sheep normally maintained on hay (total VFA, □—□; formate, ■—■) and hay with added casein (total VFA, ○—○; formate, ●—●).

rumen (Annison, 1954) were observed but, under normal conditions, these acids account for less than 1% of the total VFA level.

The distribution of blood VFA's between cells and plasma. The distribution of the component VFA's in sheep blood between cells and plasma was examined. Venous blood (100 ml.) was carefully withdrawn to avoid haemolysis and 20 ml. were examined for VFA's. The haematocrit of the blood was determined by the procedure of Meyerstein (1942) by centrifuging at 2500 *g.* for 25 min., and 70 ml. of blood were centrifuged simultaneously to separate cells and plasma. The VFA's of the cells and plasma were determined by the procedure described for whole blood (Table 4). It was assumed that the cells contained 5% (v/v) entrained plasma and corrections to the haematocrit (30·9%), cell volume and cell VFA value were applied. Considerably more formate was present in cells than in plasma. Similar results were obtained with ox blood (Table 4). In further experiments, cells were repeatedly washed with 0·9% saline and then dialysed against 20 vol. of saline at 2° for 24 hr. The VFA content of the cells was compared with that of the original blood. A significant amount of VFA was present in the washed cells, and the proportion of formate was considerably higher than that of whole blood (Table 4).

VFA content of other body fluids

VFA content of sheep saliva. Samples were obtained from sheep under normal conditions and also when anaesthetized with Nembutal. Blood samples were withdrawn for VFA analysis during the period in which saliva was collected. The results shown in Table 5 indicated the absence of formate from saliva, although formate was present in the blood samples obtained at the same time. The samples were obtained 2–4 hr. after feeding, i.e. in the period usually associated with high blood VFA levels.

VFA's of cerebrospinal fluid. Samples were obtained from two sheep on two separate occasions. In the second experiment jugular blood samples were taken at the same time and also examined for VFA's. The results shown in Table 6 indicated that the VFA level was considerably lower than in blood, but both formate and acetate were present in similar proportions to those normally found in blood.

VFA's of urine. The analysis of six samples from different sheep showed that only small amounts (0·2–0·9 m-moles/l.) were normally present. The relative amounts of formate and acetate present were similar to those usually observed in blood and the total VFA levels of both body fluids were of the same order. Traces (<1% of the total VFA's) of propionate were occasionally observed in sheep urine. In further experiments, urine samples were taken for analysis at intervals throughout 24 hr. periods and the results of a typical experiment are shown in Table 7. The daily output of VFA in the urine of sheep was found to be within the range 0·15–1·5 m-moles.

Table 4. *Distribution of blood VFA's between cells and plasma*

Animal	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
			Formate	Acetate	Propionate
Sheep	Cells	0·77	13	84	3
	Plasma	1·03	4	94	2
	Whole blood	0·96	7	90	3
Ox	Cells	0·54	11	89	0
	Plasma	0·80	9	91	0
	Whole blood	0·74	5	95	0
Sheep	Washed cells	0·24	52	48	0
	Whole blood	1·04	24	75	1

Table 5. *VFA content of blood and saliva samples taken simultaneously from sheep anaesthetized with sodium pentobarbitone (Expts.* 1 *and* 2*), and under normal conditions* (3 *and* 4)

Expt.	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
			Formic	Acetic	Propionic
1	Saliva	2·1	0	98	2
	Carotid blood	1·7	7	91	2
2	Saliva	0·8	2	95	3
	Carotid blood	1·4	7	92	1
3	Saliva	0·6	0	98	2
	Venous blood	1·2	12	86	2
4	Saliva	0·8	0	100	0
	Venous blood	0·9	14	84	2

Bastrup (1947) has reported the occurrence of relatively large amounts (0·43–4·8 m-moles/l.) of formate in dog urine and this finding was investigated using the methods described for the analysis of sheep urine. Six samples were obtained from different dogs (mixed breeds) at various times after feeding. The animals were fed once daily on rations of horse flesh (1 part) and flaked maize (3 parts) with small supplements of cod-liver oil and minerals. The total VFA concentration in dog urine appeared to be of the same order as that found in sheep urine (Table 8), but the proportion of formate was higher than that normally found in sheep urine.

Examination of alimentary tract contents for VFA's. Samples of rumen and abomasal contents were taken from two sheep fitted with permanent fistulae in the rumen and in the anterior part of the duodenum just behind the pyloric sphincter. A further sheep fitted with fistulae in the caecum and ileum was available, and samples were obtained from both organs. In another experiment, the gut from a freshly slaughtered sheep was immediately tied off into the following sections: (*a*) duodenum and jejunum, (*b*) ileum (*c*) caecum, and (*d*) the upper third of the colon. This experiment was repeated, but attention was confined to the small intestine, which was divided roughly into duodenum and jejunum, and ileum. Samples were taken from each section, and these and the other alimentary tract contents were analysed for VFA's. Liquid samples were mixed with an equal volume of N-H_2SO_4 saturated with $MgSO_4$, $7H_2O$ and semi-solid material was treated with an equal weight of the same reagent. The suspensions were filtered and 2–5 ml. of filtrate were distilled in the Markham still. The distillates were titrated with 0·01 N-NaOH under CO_2-free conditions and retained for chromatographic analysis.

Appreciable quantities of formate were present only in the small intestine (Table 9), where the total amounts of formate found in these experiments were in the range 0·8–1·0 m-mole.

VFA's of gastric juice. Four samples were collected over 30 min. periods for 2 hr. after the animal had been fed 500 g. of fresh lucerne. Since the concentrations of VFA were low, the distillates

Table 6. *VFA content of cerebrospinal fluid (c.s.f.) of sheep*

Expt.	Sheep	Sample	Total VFA (m-moles/l.)	Molecular proportions of VFA's		
				Formate	Acetate	Propionate
1	8	C.s.f.	0·28	18	82	0
	23	C.s.f.	0·16	10	90	0
2	8	C.s.f.	0·55	19	81	0
	8	Blood	1·90	—	—	—
	23	C.s.f.	0·46	25	75	0
	23	Blood	1·05	16	83	1

Table 7. *VFA content of sheep urine collected during a 24 hr. period*

Animal fed once daily (600 g. hay/day). Sampling commenced after 24 hr. fasting, and animal fed 6 hr. after experiment started.

Sampling time (hr.)	Vol. of urine (ml.)	Total VFA (m-moles/l.)	Molecular proportions of VFA's	
			Formic	Acetic
1	25	0·18	9	91
2	15	0·17	12	88
3	40	0·22	17	83
4·5	53	0·22	—	—
5·5	42	0·28	21	79
6·5	24	0·28	15	85
7·5	33	0·28	—	—
9·0	13	0·28	0	100
11·5	60	0·19	—	—
14·5	115	0·21	8	92
24·0	120	0·24	11	89

Table 8. *VFA content of dog urine*

Dog	Sex	Sampling time (hr. after feeding)	Total VFA (m-moles/l.)	Molecular percentages of VFA's		
				Formic	Acetic	Propionic
1	m	1	0·40	53	47	0
2	m	1	0·60	57	43	0
3	f	2	1·09	62	38	0
4	m	4·5	0·64	48	49	3
5	m	23	0·53	51	49	0
6	m	1	0·68	53	47	0

from the four samples were pooled for chromatographic analysis. Formate and acetate were present in similar proportions to those usually found in blood, although the total VFA levels were much lower than those of blood (Table 10).

VFA utilization by the tissues

The mammary gland of the goat. A lactating goat prepared with both an exteriorized carotid artery and an exteriorized mammary vein by Dr J. L. Linzell for another purpose was used in these experiments. Blood samples from the carotid artery and mammary vein were withdrawn simultaneously during the recording of arterial blood pressure while the animal was standing quietly. The results of VFA analyses on these samples, together with analyses of blood gases, sugar and haemoglobin supplied by Dr Linzell, are shown in Table 11. These figures corroborate that the animal was undisturbed and show that milk secretion was taking place. In the excited animal the uptake of milk precursors may cease (Graham, Kay & McIntosh, 1936) or the blood may become concentrated or diluted during its passage through the gland (Powell & Shaw, 1942). The results of the blood VFA analyses indicate that only a small amount of formate was taken up by the udder, although the acetate level fell 0·7–1·2 m-moles/l. McClymont (1951*b*) reported the uptake of acetate (0·2–1·0 m-mole/l.) from blood by the lactating bovine udder.

Table 9. *VFA content of various sections of the alimentary tract of the sheep*

			Molecular proportions of VFA's						
Expt.	Sample	Total VFA (m-moles/l.)	Formic	Acetic	Propionic	*iso*-Butyric	Butyric	*iso*Valeric and 2-methyl-butyric	*n*-Valeric
1	Rumen	83	0	57	21	2	14	3	3
	Abomasum	5	2	72	16	2	6	1	1
2	Rumen	97	1	66	20	2	8	2	1
	Abomasum	8	1	80	14	1	4	0	0
3	Duodenum and Jejunum	0·8	14	76	7	0	3	0	0
	Ileum	3·8	22	77	1	0	0	0	0
	Caecum	19·5	1	72	16	3	5	2	1
	Colon	6·5*	0	72	16	2	6	2	2
4	Duodenum and Jejunum	7·0	22	73	3	0	1	0	1
	Ileum	12·0	21	73	3	0	2	0	1
5	Ileum	3·3	19	70	8	1	2	0	0
	Caecum	100	2	72	15	2	6	1	2
6	Ileum	4·0	7	84	9	0	0	0	0
	Caecum	20	1	78	15	1	3	1	1

* In m-moles/kg.

Table 10. *VFA content of sheep gastric juice*

Sampling period (min. after feeding)	Volume collected (ml.)	Total VFA (m-moles/l.)	VFA content of pooled samples	
			Formate	Acetate
5–35	16	0·30	18	82
35–65	15	0·22		
65–95	14	0·22		
95–125	14	0·21		

Table 11. *The VFA's haemoglobin and sugar content of blood withdrawn simultaneously from the carotid artery and mammary vein of a lactating goat, and respiratory quotients calculated from the blood gases of the samples*

Expt.	Sample	Total VFA (m-moles/l.)	Formate (m-moles/l.)	Acetate (m-moles/l.)	Haemoglobin (g./100 ml.)	Sugar* (mg./100 ml.)	Respiratory quotient
1	Carotid	1·3	0·12	1·18	9·62	53	1·50
	Mammary vein	0·5	0·12	0·37	9·55	38	
2	Carotid	1·3	0·16	1·14	9·50	52	—
	Mammary vein	0·6	0·12	0·41	9·55	36	
3	Carotid	1·7	0·20	1·47	9·05	53	1·97
	Mammary vein	0·5	0·13	0·36	9·25	31	

* Determined by method of Shaffer & Hartmann (1920).

Table 12. *VFA content of samples of venous (jugular) and arterial (carotid) sheep blood withdrawn simultaneously*

Time after feeding (hr.)	Total VFA (m-moles/l.)		Molecular proportions of VFA's					
			Formic		Acetic		Propionic	
	Art.	Ven.	Art.	Ven.	Art.	Ven.	Art.	Ven.
Pre-feed	0·68	0·56	16	21	82	77	2	2
3	1·87	1·43	10	6	88	93	2	1
5	1·97	1·22	7	13	93	86	0	1
17	1·45	1·17	6	7	92	91	2	2

Table 13. *Analysis of blood VFA's of several animal species*

Animal	No. of samples examined	Source of blood	Range of total VFA's (m-moles/l.)	Individual VFA levels (molecular proportions)		
				Formic	Acetic	Propionic
Horse	3	Venous	0·27–0·96	4–45	54–73	1–4
Dog	6	Venous	0·18–0·48	31–53	45–66	1–4
Rabbit	3	Arterial	0·89–1·55	6–30	70–90	0–4
Cat	3	Arterial	0·26–0·46	36–54	41–60	3–5
Goat	6	Venous	0·51–1·61	10–30	69–88	0–2
Cattle	3	Venous	0·55–1·32	12–28	71–87	0–3
Human	5	Venous	0·28–0·32	23–34	64–77	0–4

The tissues of the head of the sheep. Carotid artery and jugular vein samples were taken simultaneously from a sheep with an exteriorized carotid artery at intervals after feeding and analysed for VFA's. The results of a typical experiment are shown in Table 12.

The uptake of appreciable quantities of acetate in some cases was indicated, confirming the results obtained by Reid (1950) in similar experiments, but it would appear that little, if any formate is utilized or produced by the tissues of the head.

Blood VFA's of other species

The blood of a number of other species was examined in order to extend the findings made with sheep blood. Complete VFA analyses were made on human blood (5 samples) and on the blood of dogs (6), rabbits (3), cats (3), horses (3), goats (6) and cattle (3). Formate, in addition to acetate, was present in every sample examined. Small amounts of propionate were also observed (Table 13). The occurrence of formate in blood is obviously not confined to ruminants.

DISCUSSION

The occurrence of appreciable amounts of formate in every blood sample examined, irrespective of source, was an unexpected finding since many recent investigations of the nature of blood VFA's have made no mention of this acid as a blood constituent (cf. McClymont, 1951*a*; Craine & Hansen, 1952). Although the chromatographic findings were confirmed by the isolation of formic acid from sheep blood, the possibility still exists that a precursor in blood gives rise to formic acid under the acid conditions employed either to steam distil the deproteinized blood filtrates or to extract the blood VFA's with ether before chromatographic analysis.

The concentration of formate in sheep blood appeared to be fairly constant, since although the blood acetate rises sharply after feeding, due to increased acetate production in the rumen, there is little change in the level of formate. Examination of the blood VFA's of fasted sheep indicated that the formate concentration in blood fell only slightly during a 48 hr. fasting period, although the acetate levels were greatly reduced.

Analysis of the contents of each part of the alimentary tract of the sheep indicated that significant amounts of formate were present only in the small intestine. Since formate is virtually absent from abomasal contents, the acid must either (*a*) be present in one or more of the secretions into the small intestine, or (*b*) be liberated as a result of the chemical or enzymic processes which occur in this organ, or (*c*) arise by bacterial action. The latter possibility is unlikely, since the environment is not favourable for bacterial activity. The total amount of formate present in the small intestine is probably not sufficient to account for the major part of the blood formate unless the acid is rapidly absorbed into the circulation.

The absence of formate from sheep saliva was an unexpected finding in view of the presence of small amounts of acetate (0·3–2·1 m-moles/l.). It would appear that either formate is utilized by the secreting salivary glands, or that there is a threshold value for formate under normal conditions since, when formate is administered intravenously to sheep, the acid can be detected in the saliva (unpublished results). Salivary secretion in the

ruminant is of importance in the maintenance of fluid and mineral levels in the rumen, and the daily output of saliva is probably of the order of 4 l. (Scheunert & Trautmann, 1921). The results obtained for the acetate concentration in sheep saliva in the present studies suggests that 2–8 m-moles of acetate are returned each day to the rumen in this manner: this amount of acetate is not significant when compared with the acetate concentration in the rumen (50–100 m-moles/l.).

An examination of sheep urine showed that small amounts of formate and acetate were normally present. The daily output of VFA (0·15–1·5 m-moles) represents a negligible energy loss to the animal. The total VFA content of dog urine (0·4–1·0 m-moles/l.) was similar to that of sheep urine, but the proportions of formate and acetate were almost equal and similar to the relative concentrations in dog blood. The formate levels in the samples of dog urine examined (0·21–0·6 m-mole/l., i.e. 1·0–3·1 mg./100 ml.) were lower than those reported by Bastrup (2–22 mg./100 ml.) in 1947.

The toxic effects of methanol have been attributed to the formate produced by the oxidation of this substance in the tissues (cf. Wilson & Schild, 1952). The demonstration of formate as a normal blood constituent suggests that although this acid is undoubtedly produced by the *in vivo* oxidation of methanol (Kendall & Ramanathan, 1953) it is not responsible for the toxicity of this alcohol. In recent experiments (Annison & Hill, to be published) aqueous solutions (100–150 ml.) of sodium formate (5–8 m-moles/kg. body wt.) were administered to sheep by injection into the jugular vein over periods of 10–20 min. The animals showed no ill-effects and the blood formate level returned to normal in 3–4 hr. The collection and analysis of urine after the administration of formate indicate that about 20% of the acid was excreted in the urine during this period.

Formate metabolism in animal tissues has been the subject of many recent investigations. The utilization of formate in the synthesis of serine (Sievekitz & Greenberg, 1949; Kruhøffer, 1951), purines (Sonne, Buchanan & Delluva, 1948; Greenberg, 1951) and labile methyl groups (Sakami & Welch, 1950; du Vigneaud, Verly & Wilson, 1950; Berg, 1951) has been demonstrated. Berg (1953) has shown that pigeon-liver extracts incorporate [^{14}C]formate into the methyl groups of methionine, cystathionine, the β-carbon of serine and the 2- and 8-carbons of purines. Sperling, Maxwell & Oettingen (1953) injected [^{14}C]formate into rats and measured the excretion and distribution of radioactive carbon. A considerable portion of the radioactivity (80%) was excreted within 24 hr., largely as $^{14}CO_2$. Examination of the tissues a week later indicated that the depot fats and proteins possessed measurable activity. Formate is obviously readily utilized by animal tissues, and the fairly constant levels observed in sheep blood suggest that the acid is continuously produced by other metabolic processes. The present studies have been largely confined to ruminants, but the results shown in Table 12 indicate that the concentration of formate in the blood of each animal species investigated is of the same order.

SUMMARY

1. A method has been developed for the complete analysis of the volatile fatty acids (VFA's) present in 10–20 ml. of blood.

2. Formic acid has been identified as a constituent of sheep blood and normally accounts for 10–30% of the molecules of VFA present.

3. Chromatographic evidence was obtained indicating that formic acid was present in human blood and in the blood of goats, cattle, horses, rabbits, cats and dogs at concentrations similar to those observed in sheep blood.

4. No evidence was obtained for the uptake or production of appreciable amounts of formate by the tissues of the actively secreting mammary gland of the goat, or of the head of the sheep.

5. The examination of the VFA's found in the alimentary tract of the sheep suggested that the formate in the peripheral circulation was probably not derived from this source.

6. Small amounts of VFA were demonstrated in sheep urine (0·17–0·28 m-moles/l.). The relative proportions of formate and acetate were similar to those observed in blood. The daily output of VFA in the urine was in the range 0·15–1·5 m-moles, which represents a negligible energy loss to the animal. Larger amounts of VFA were found in dog urine (0·40–1·09 m-moles/l.), and the proportions of formate and acetate were similar to those observed in dog blood, i.e. approximately 1:1.

7. Investigation of the distribution of blood VFA's between cells and plasma in sheep and cattle indicated that formate is considerably concentrated in the cells.

The author wishes to thank Dr I. W. McDonald and Mr K. J. Hill for the preparation of the experimental animals, and Dr J. L. Linzell and Mr Hill for supplying samples of body fluids from a number of animals. The advice of Drs S. R. Elsden and A. T. Phillipson is gratefully acknowledged.

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The Estimation of Serum Lipoproteins. A Micromethod Based on Zone Electrophoresis and Cholesterol Estimations

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(*Received* 29 *June* 1954)

Investigations in recent years have indicated that the lipids do not exist 'free' in serum, but are present in combination with protein; the position has been adequately summarized by Russ, Eder & Barr (1951). The development of physical methods in recent years allowed many different approaches towards elucidation of the nature of these complex serum lipoproteins.

The elegant low-temperature ethanol fractionation procedure applied to plasma by Cohn and co-workers demonstrated that almost all the plasma lipids could be accounted for in two separate protein fractions, termed IV.1 and III.0 (Gurd, Oncley, Edsall & Cohn, 1949; Oncley, Gurd & Melin, 1950). These fractions contained largely the α- and β-globulins respectively and hence the lipid fractions were termed the α- and β-lipoproteins.

A second approach to the problem was produced by the ultracentrifuge studies of Gofman, Lindgren & Elliott (1949). Subsequent comprehensive studies from this school (Lindgren, Elliott & Gofman, 1951) suggest that by this procedure the β-lipoprotein component can be shown to be a mixture of molecular species of varying densities.

A third method for the study of serum lipoproteins is zone electrophoresis as developed by Wieland & Fischer (1948), Durrum (1950), Turba & Enenkel (1950), Cremer & Tiselius (1950), Kunkel & Slater (1952). This recent, but already well-known technique, permits isolation of each electrophoretic component in a fairly pure state. Thus zone electrophoresis should be an ideal method for the study of protein–lipid relationships in serum.

Fasoli (1952) separated the plasma proteins by paper electrophoresis using the method of Flynn & de Mayo (1951) in which the paper was stained with sudan III, and showed the presence of two lipid bands corresponding with the α- and β-globulins. Roughly the same results were obtained by Rosenberg (1952) employing sudan IV as the staining reagent, while Durrum, Paul & Smith (1952) used oil red O, and Swahn (1952, 1953) used sudan black.

Kunkel & Slater (1952) separated the lipoproteins from 2 to 5 ml. sera by zone electrophoresis in a starch block and analysed the various fractions for cholesterol and phospholipids. This method confirmed the presence of the two major lipoprotein constituents, and the results compared favourably with the lipid-staining techniques.

The purpose of the present study was to investigate the possibilities of zone electrophoresis on filter paper using about 0·1–0·2 ml. serum, followed by serial cholesterol determinations on the paper segments. The lipoprotein pattern could then be reconstructed from the cholesterol estimations. After completion of this work, a method based on the same general principle was published by Nikkila (1953), but differences exist in the electrophoresis apparatus, the technique and the micro cholesterol method used in this investigation.

MATERIALS AND METHODS

Apparatus. The supporting medium used in the electrophoresis run was Whatmann 3 MM filter paper, 14 × 5 in. The filter paper was clamped taut between two glass plates each 9 × 6 × ⅛ in. but prevented from touching the plates by four Perspex distance pieces each 6 × ½ × ¼ in. A screw-clamping device allowed the lower glass plate to press on a heavy brass cooling plate 8 × 5 × ¼ in., which had an optically flat upper surface. A long coil of ¼ in. copper tubing was soldered to the lower surface of the cooling plate, to permit the circulation of tap water through the apparatus. The whole apparatus was then covered with an inverted glass jar.

The buffer was 0·04 M diethyl barbituric acid–sodium diethyl barbiturate, pH 8·6. The Perspex electrode vessels each contained 500 ml. buffer, and the electrodes were platinum strips 4 × ½ in. The hydrostatic pressure in the vessels was equilibrated before each run by means of a glass bridge placed temporarily between the troughs.

Method. A pencil line was drawn across a piece of Whatman 3 MM, 14 × 5 in. about 4 in. from one end. Buffer was sprayed on to this line producing a damp band about 1 in. wide. Using a drawn-out capillary pipette, 0·15 ml. serum was evenly applied to the line to within ¼ in. from either end. The remainder of the paper was then sprayed with the buffer solution, with the exception of about 2 in. at each end which were left dry in the meantime to facilitate the insertion of the paper in the apparatus. After clamping tightly in the apparatus with the thumb screws, the ends of the paper were dipped in the buffer solutions. At this stage, with cooling water running through the apparatus, a period of 30 min. was allowed for temperature and hydrostatic equilibration.

A potential difference of 300 v was then applied across the paper, the current carried by the paper being about 6 ma. The air temperature within the case was controlled to 12 ± 1°, the period of each run was 8 hr. Upon completion of the run, the paper was removed, and allowed to dry in air for several hours. Segments 1 cm. apart were marked out on the paper, parallel to the line of origin, and then a piece about 9 × ½ in. cut lengthwise from the centre of the filter paper, to be used later for qualitative protein detection.

Cholesterol-extraction procedure. The paper was then cut into 1 cm. pieces, combining corresponding segments from either side of the portion which had been removed for protein staining. Thus two 5 × 1 cm. pieces of filter paper were inserted into each centrifuge tube and 7 ml. acetone–ethanol (1:1, by vol.) added. The tubes were placed in a water bath at about 30° and the temperature was gradually raised until it reached 66°, where it was maintained for 10 min. and then allowed to cool to 40°.

The filter-paper strips were removed from the solvent by stainless-steel forceps and each strip was washed with a fine jet of acetone–ethanol, adding the wash liquid to the extract. The centrifuge tubes were placed in a water bath at 60° and the solvent was completely removed by a gentle stream of air. A thin glass rod was placed in each tube, and all the tubes were set in a beaker of sand in an oven at 110° for 30 min.

Cholesterol estimation. Each tube was then treated with 0·5 ml. A.R. glacial acetic acid, followed by 1·0 ml. freshly prepared ice-cold acetic anhydride–sulphuric acid reagent (20:1, by vol.). The contents of each tube were thoroughly stirred and incubated at 25° in the dark for exactly 30 min. The resultant coloured solutions were transferred by dry Pasteur pipettes to a glass cell of capacity 1 ml. and light-path 2 cm. and the optical density of each solution was read in a Spekker absorptiometer using Ilford filter no. 607 (max. transmission 580 mμ. onwards). The cholesterol contained in each sample was determined from a calibration graph prepared from standard cholesterol solutions.

Plotting the amount of cholesterol found per segment against the migration distance from the origin yields the lipoprotein pattern. A typical example of a pattern shown by normal serum is shown in Fig. 1. For comparison, the result of the qualitative protein staining from the same run is shown on the figure also. The bromophenol blue method of Durrum (1950) was used in the latter procedure.

It was shown that the presence of diethylbarbituric acid did not affect the cholesterol colour reaction, hence the small quantity of buffer eluted from the paper by the lipid solvent would not influence the subsequent cholesterol determination.

Calculation of α-:β-lipoprotein ratio. A line was drawn on each lipoprotein pattern parallel to the base-line in such a manner that both the cholesterol (lipoprotein) peaks were isolated from as much 'background' material as possible. This is shown as line A–A_1 on Fig. 1. From this datum line, the area under each component was calculated, and the cholesterol on the α-lipoprotein expressed as a percentage of the total cholesterol on both the α- and β-lipoproteins.

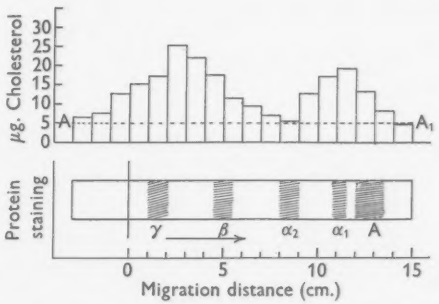

Fig. 1. Serum lipoprotein pattern (*a*) contrasted with qualitative protein staining from same paper (*b*). For details of electrophoresis and cholesterol see text. The qualitative staining for protein was done with bromophenol blue.

EXPERIMENTAL

Experiments performed to justify the procedure

The initial experiments on the separation of the lipoproteins were performed using the 'hanging paper' method of Flynn & de Mayo (1951), but serial lipoprotein results were disappointing in their reproducibility. This was thought to be due to the buffer gradient throughout the paper, because upon changing to the filter-paper technique of Kunkel & Tiselius (1951) the results became more reproducible. In the latter technique the paper is clamped horizontally between two glass plates, thus eliminating the buffer concentration gradient effect, and also the evaporation difficulties. However, while it was possible to achieve resolution of the lipoproteins by this method, a difficulty arose in that the lipoprotein component bands and the bands due to the qualitative staining of protein were more diffuse by this method than when run free in air. In an attempt to obtain the advantages of both methods, the procedure previously outlined was adopted, namely to run the filter paper horizontally without touching the glass plates. Fig. 2 shows the lipoprotein pattern obtained from a normal serum estimated by the method outlined, but *a*, run in air (Flynn & de Mayo, 1951); *b*, run between glass plates (Kunkel & Tiselius, 1951); and *c*, run horizontally in air. All were run under the same conditions of buffer ionic strength, pH, time, temperature, etc.

These results showed that the lipoprotein pattern appeared much sharper when the paper was run in air.

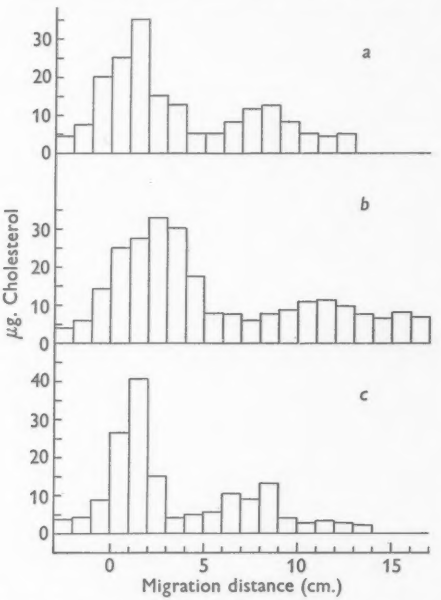

Fig. 2. Comparison of serum lipoprotein patterns obtained by electrophoresis according to: *a*, Flynn & de Mayo (1951); *b*, Kunkel & Tiselius (1951); and *c*, the present method. *a*, run in air; *b*, run between glass plates; *c*, run horizontally in air. For other details see text.

Even treatment of the glass plates with silicone grease failed to improve significantly the definition of the pattern. Nevertheless, in a series of sera run horizontally between silicone-treated glass plates, and horizontally in air, the α-:β-lipoprotein ratios were not significantly different. The sharper definition of the lipoprotein patterns obtained by the 'air gap' method decided in favour of this technique.

Ionic strength of the buffer. Under the conditions outlined in the method, with buffer of $I = 0{\cdot}10$, an electrophoresis run of 8 hr. failed to separate completely the α- and β-lipoproteins. As the ionic strength of the buffer decreased the resolution of these entities increased. However, at the lowest value tried, $I = 0{\cdot}02$, the pH of the buffer was difficult to control and for this reason the concentration found most suitable was $I = 0{\cdot}04$.

Change in buffer pH *during electrophoresis.* Employing 500 ml. buffer of $I = 0{\cdot}04$ in each vessel, the change in hydrogen-ion concentration between the anode and the cathode vessels was less than 0·05 pH unit/hr. This showed that the ionic strength of the buffer and the capacity of the vessels were adequate for the electrophoresis period used.

The effect of temperature on the lipoprotein pattern. The influence of temperature on the lipoprotein separation was studied, and it was found that there was little difference in the lipoprotein patterns obtained at 1, 8 or 12°; above this last temperature there was a steady deterioration in the resolution of the α and β fractions.

Thus for the purpose of this investigation, cold water from the mains was passed through the cooling device in order to keep the temperature within the case as constant and low as possible, i.e. $12 \pm 1°$.

Paper temperature during electrophoresis. Since the temperature recorded by the above procedure was the air temperature, it was thought advisable to study the changes in temperature (if any) which occurred along the paper and throughout a normal run of 8 hr. For this purpose a thermistor device (Standard Telephones and Cables Stantel Type U 2361/20) and galvanometer were used coupled to a temperature-calibrated Wheatstone bridge. Using the electrophoresis apparatus as detailed above the temperature variation along the paper was negligible. A slight drop in temperature at the centre of the paper was due to the sag in the paper which brought the latter closer to the cooling plate assembly. During an 8 hr. run there was no appreciable change in temperature at the centre of the paper.

Rate of migration of lipoproteins. The rate of migration of the lipoproteins had to be studied in order to define the optimum time of electrophoresis. It was found that a 6 or 8 hr. run caused effective separation of the α- and β-lipoproteins, while 12 hr. and over made the patterns diffuse. In order to obtain maximum resolution within as short a run as possible the period of 8 hr. was selected.

Comparison of the lipoprotein patterns obtained from plasma, serum, and dialysed serum. A comparison was made of the lipoprotein patterns obtained by this method, of oxalated plasma, serum, and serum dialysed for 48 hr. at 3° against 0·04M diethyl barbiturate buffer, all obtained from the same blood specimen.

The results are shown in Fig. 3. While the α-:β-lipoprotein ratios in these preparations were similar, the shape of the β-lipoprotein peak was altered. For instance, in serum there appeared to be less 'adsorption' of cholesterol near the origin, producing a more symmetrical β-lipoprotein peak with less tailing. Presumably this was due to

removal of fibrin, which tends to be deposited at the origin, possibly trapping some lipid matter.

The lipoprotein patterns of the dialysed sera samples differed little from the corresponding non-dialysed samples, and the α-:β-lipoprotein ratio was unaffected by this procedure. Thus subsequent studies were performed with non-dialysed sera.

Comparison of the lipoprotein pattern obtained with 1 *cm. and* 0·5 *cm. segments.* In order to establish whether the 1 cm. segment cuts were of a suitable size to give an accurate representation of the lipoprotein pattern the following procedure was adopted. A number of sera were run in duplicate under standard conditions, one paper being cut into 1 cm. and the other into 0·5 cm. segments. The cholesterol was eluted and estimated in the usual manner. It was found that similar lipoprotein patterns were obtained by each method. Thus there seemed to be no advantage in using the smaller segments, and in fact the disadvantage that the number of cholesterol estimations was doubled while the quantity of cholesterol per segment was halved. This experiment also settled any doubt that the lipoprotein pattern produced by the 1 cm. segment method might have had a 'fine structure' which was being obscured by the magnitude of these cuts.

Examination of various types of supporting media. Whatman nos. 1, 4 and 3 MM papers were compared, and for the purpose of this lipoprotein study, Whatman 3 MM was found to be most suitable. This paper, 5 in. wide (effective width about 4·5 in.) could easily accommodate 0·15 ml. serum as a narrow band, and the paper had little (if any) lipid-soluble matter. The lipoprotein pattern obtained on Whatman 3 MM filter paper was contrasted with the pattern obtained on the same type of paper which had been extracted for 16 hr. in a Soxhlet apparatus with ether and dried at 60° before use. The serum lipoprotein pattern was unaffected by prior ether extraction of the supporting medium; furthermore the small 'background' material present in all the estimations was not decreased by this procedure. Thus for normal use, Whatman 3 MM filter paper of 'For Chromatography' grade was used throughout without any pretreatment.

To test the efficiency of the cholesterol-extraction technique. The paper segments after cholesterol extraction in the usual manner were combined and extracted with ether in a Soxhlet apparatus for 12 hr. The extracts were taken to dryness, transferred to centrifuge tubes with acetone–ethanol, and cholesterol estimated by the micromethod as detailed previously.

In eight lipoprotein estimations the mean residual unextracted cholesterol amounted to 5·1%. Thus the 95% efficiency of the simple centrifuge-tube extraction procedure was considered quite adequate. Changing to chloroform–methanol (1:1, v/v) failed to improve the efficiency of the extraction and consequently acetone–ethanol was adopted as the solvent of choice.

Comparison of this method of lipoprotein estimation with a lipid-staining technique. Eight lipoprotein estimations were run in the usual manner, but after removal of the centre section of each paper (for qualitative protein staining) only one half was employed in the cholesterol estimations. The remaining portion of each paper was stained with sudan IV employing the method of Rosenberg (1952). Qualitatively the α- and β-lipoprotein bands on the stained paper coincided exactly with the two cholesterol peaks when the latter results were plotted on the same scale. A typical example of this is shown in Fig. 4.

Reproducibility of the lipoprotein method. In order to establish the degree of reproducibility of the method the following experiment was conducted. A sample of serum was obtained from a normal subject and twenty lipoprotein estimations were performed on this single sample over a period of 11 days. The serum was stored meanwhile in the refrigerator. The α-:β-lipoprotein ratio was calculated for

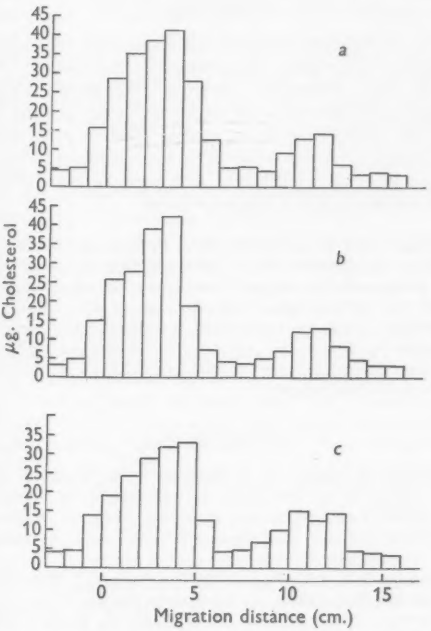

Fig. 3. Comparison of the lipoprotein patterns obtained from: *a*, plasma; *b*, serum; and *c*, dialysed serum. For further details see text and Figs. 1 and 2.

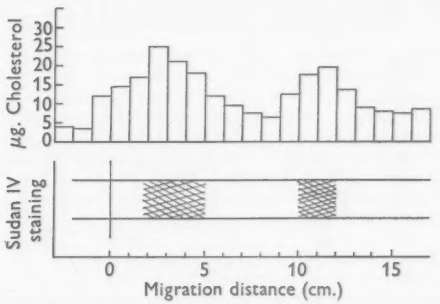

Fig. 4. Comparison of the lipoprotein pattern obtained by the method described and a pattern obtained by a sudan IV staining method.

each sample, and the mean values and standard deviations of this group were as follows:

$$\alpha:\beta::34\pm3{\cdot}6:66\pm3{\cdot}6.$$

This degree of precision appeared adequate for present purposes.

Chemical separation of the lipoproteins followed by electrophoresis of the components. As a final check on the method the α- and β-lipoproteins were separated by the Cohn fractionation procedure (Cohn *et al.* 1950). The unfractionated serum, fractions (I + II + III) and fractions (IV + V) were submitted to zone electrophoresis followed by lipoprotein estimation in the usual way. The results (Fig. 5) showed that the serum α-lipoprotein occurred in fractions (IV + V) while the β-lipoprotein appeared in fractions (I + II + III). Thus lipoprotein data obtained by this procedure should correspond with those obtained by the Cohn method.

DISCUSSION

This method for the estimation of serum α- and β-lipoproteins is relatively simple, cheap, and yields very reproducible results. Under the electrophoresis conditions described, it is possible to separate the two main lipoproteins, without separating the 'α-' lipoprotein into its main α_1 and lesser α_2 fractions. The concentration of each lipoprotein is assessed from the amount of cholesterol associated with it and this quantity is derived from the summation of serial micro cholesterol determinations. The various check experiments outlined in the text suggest that the conditions chosen for this separation approximate to the ideal.

A possible slight source of error in the calculation of the α-:β-lipoprotein ratio is due to the method of cholesterol estimation. The ratio of free cholesterol to ester cholesterol is lower in the α-lipoprotein than in the β-lipoprotein (Kunkel & Slater, 1952), and since ester cholesterol gives a more intense colour in the Lieberman–Burchardt reaction, (Gardner & Williams, 1921) this will tend to increase the 'α' component owing to its higher ester content. This small error, while interfering with the calculation of the absolute ratio of α-:β-lipoprotein, does not detract from the usefulness of the technique as a comparative method. An advantage of this micromethod for the estimation of serum lipoproteins lies in the very small quantity of blood required—a finger-prick sample would suffice.

The sera used in this study were from normal fasted subjects. The effect of chylomicra on the lipoprotein pattern, and the variations of these patterns under physiological and pathological conditions will be reported elsewhere.

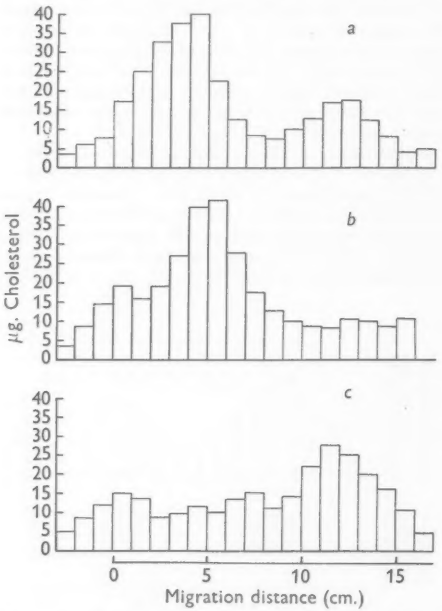

Fig. 5. Comparison of lipoprotein patterns obtained from: *a*, unfractionated serum; *b*, Cohn fraction (I + II + III) (i.e. β-lipoprotein); and *c*, Cohn fraction (IV + V) (i.e. α-lipoprotein).

SUMMARY

1. A micromethod for the separation and estimation of the serum lipoproteins is described.

2. The procedure involves filter-paper zone electrophoresis of serum, followed by segmentation of the supporting media, elution of cholesterol and estimation of the latter by a micromethod. Summation of the cholesterol data permits calculation of the ratio of α- to β-lipoproteins.

The author is indebted to Mr J. F. Syme and Mr W. C. Cooper for skilled technical assistance. This work is part of a programme of research undertaken on behalf of the Coronary Thrombosis Study Group of the Standing Medical Advisory Committee (Scotland). The author wishes to thank the Advisory Committee on Medical Research (Scotland) for a grant from which the expenses of the work were defrayed.

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The Activation of Aconitase by Ferrous Ions and Reducing Agents

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(*Received* 17 *March* 1954)

Dickman & Cloutier (1951) showed that aconitase is dependent on the presence of ferrous ions and a reducing agent for maximum activity. Qualitative studies indicated that the ferrous ion is capable of forming complexes with the substrates of aconitase and with the enzyme under the conditions of optimum enzyme activity. The authors explained the action of ferrous ion in terms of the hypothesis advanced by Smith (1951), namely, that the metal is responsible for the linkage between the apoenzyme and the substrate in the aconitase–tricarboxylic acid complex. They assumed that the reducing agents played a double role in maintaining the iron in the ferrous state and in keeping the reducing groups of the protein in the reduced state.

The high residual activity of the aconitase preparation of Dickman & Cloutier (1951) in the absence of ferrous ion and reducing agents did not permit of a more detailed investigation of the function of these substances in the aconitase system. The aconitase preparation obtained from pig heart by the method of Morrison (1954*a*) provided a means of doing this. The preparation showed little activity in the absence of ferrous ion and a reducing agent, whereas it showed considerable activity on the addition of these substances. This preparation was used for testing the effect of ferrous ion and reducing agents on the aconitase activity. The results reported in this paper are consistent with the idea that both ferrous ion and the reducing agents act as activators or prosthetic groups of aconitase and are thus concerned in the formation of the active enzyme complex. It is suggested that the active form of the enzyme may be an enzyme–Fe^{2+}–reducing agent or an enzyme–Fe^{2+}–activator complex. For a preliminary communication, see Morrison (1954*b*).

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EXPERIMENTAL

Methods

Details of the methods for the preparation and activation of aconitase as well as the determination of enzymic activity have been previously described (Morrison, 1954*a*). Electrophoretic analysis of the final enzyme preparation showed that the major component, which possessed aconitase activity, formed approximately 75% of the total protein present. After dialysis against 0·004 M citrate buffer, pH 5·7, the enzyme could be stored in the frozen state for many weeks with only slight loss of activity. After activation with 5×10^{-4} M-Fe^{2+} and 10^{-2} M cysteine, 1 mg. of this preparation was capable of forming 230 μmoles of citric acid from *cis*-aconitic acid in 15 min. at 30° and pH 7·4 in the absence of buffer.

Aconitase was activated by adding a sample of the enzyme to a solution containing the indicated amounts of ferrous ammonium sulphate and cysteine, or other reducing agent. The mixture was then neutralized to pH 7·4 with N-NaOH, using a glass electrode and, except where otherwise stated, was incubated in an ice bath for 1 hr. before the determination of aconitase activity. Activation could not be carried out in the presence of a buffer on account of the ability of buffers to inactivate aconitase in the absence of substrate (Morrison, 1954*a*).

The conditions used for the determination of aconitase activity are described in the legends to the figures. Buffers were used in the later experiments for it was found that when the enzyme was added to a mixture of substrate and buffer, there was no loss of enzyme activity over the period of the test.

In all instances the reaction *cis*-aconitate→citrate was studied. Citric acid was estimated by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Materials

cis-Aconitic anhydride was prepared from *trans*-aconitic acid by the method of Malachowski & Maslowski (1928). Sodium *cis*-aconitate was formed by neutralizing the anhydride to pH 7·4 with N-NaOH. Ascorbic acid and cysteine were obtained from Roche Products Ltd., thioglycollate was a British Drug Houses Ltd. product and glutathione was a gift from the Distillers Co. (Biochemicals) Ltd., Liverpool. As determined by thiol titration, the glutathione was 99% pure (Cecil & McPhee, to be published). All these compounds were free of iron. The other reagents were A.R.

RESULTS

Activation of aconitase by ferrous ion and cysteine

Fig. 1 shows the effect of Fe^{2+} and cysteine on the activity of aconitase. The purified, dialysed preparation possesses little activity; cysteine alone does not increase the activity; Fe^{2+} brings about a 15-fold increase in the activity whilst the addition of Fe^{2+} and cysteine increases the activity 70-fold. The investigation of the activation of aconitase by Fe^{2+} alone involved some technical difficulties.

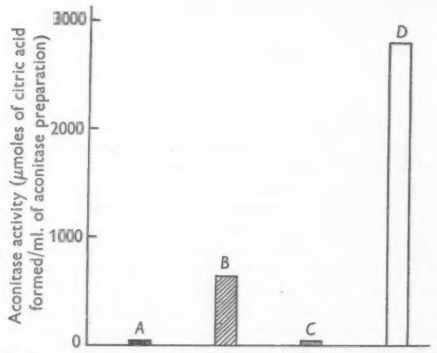

Fig. 1. Activation of aconitase by Fe^{2+} and cysteine. Activation was carried out by incubating samples of the aconitase preparation, which contained 12 mg. of protein/ml., with the indicated solutions in a final volume of 10 ml. for 1 hr. at 0° after adjustment of the pH to 7·4. The samples were equivalent to 1·0 ml. of the aconitase preparation, except when the enzyme was incubated with both Fe^{2+} and cysteine. In this instance, a 0·1 ml. sample was used. The enzymic activity was determined by adding 0·1 ml. samples of the above enzyme solutions to media containing 0·5 ml. of *cis*-aconitic acid (final concentration, 4×10^{-3}M) at pH 7·4 and 4·4 ml. of water. The reaction was stopped after 15 min. at 30° by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid. Citric acid was estimated as described in the text. *A*, no additions; *B*, 5×10^{-4}M-Fe^{2+}; *C*, 0·01M cysteine; *D*, 5×10^{-4}M-Fe^{2+} + 0·01M cysteine.

Because of the small amount of protein present in solution, it showed little or no buffering capacity. The ferrous ammonium sulphate solution was acidic so that if it were not partly neutralized the pH of the Fe^{2+}–enzyme solution fell below 5·0 with a result that there was denaturation of the enzyme. The adjustment of the pH of this solution to 7·4 was difficult and at this pH value at least some of the Fe^{2+} was in the colloidal state. On the other hand, there was no formation of colloidal iron when a reducing agent was also present and in the presence of cysteine, the solution was buffered at pH 7·4. Therefore it is not possible to state that the above value represents an accurate estimation of the activation of aconitase by Fe^{2+}. Nevertheless, it is clear that Fe^{2+} and cysteine bring about a very marked activation of the highly purified aconitase preparation. The activation was due to Fe^{2+} ions, as ammonium and sulphate ions were inert.

Factors affecting the activation of aconitase

It is clear from Table 1 that there is an initial rapid activation of aconitase by Fe^{2+} and cysteine. The activity is increased by further incubation. At 30 min. the enzyme activity is maximal. This activity is retained for at least 4 hr., after which there is a slow decrease; at 24 hr. the enzyme possesses only 60% of its maximum activity. The loss of enzyme activity could not be ascribed to alterations in the pH of the enzyme solution or to an accumulation of the end-products of the oxidation of cysteine catalysed by Fe^{2+} in the presence of oxygen. The pH was constant over the 24 hr. period of the test. When the non-activated enzyme was added to a mixture of Fe^{2+}, cysteine and water which had previously been incubated at 0° for 24 hr., the activity following a 30 min. incubation at 0° was

Table 1. *Effect of pre-incubation time on the activation of aconitase by ferrous ion and cysteine*

Aconitase was incubated at pH 7·4 and 0° with 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine. Samples (0·2 ml.), equivalent to 12 μg. of the final aconitase preparation, were removed at intervals and added to test tubes containing 4·8 ml. *cis*-aconitate (final concentration 4×10^{-3}M), pH 7·4. Reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citrate. Temp. 30°.

Time of activation (hr.)	Aconitase activity (μg. citrate formed/15 min.)
0	285
0·5	335
1·0	320
2·0	320
3·0	320
4·0	318
5·0	295
6·0	278
24·0	192

maximal. It is likely that the loss of enzyme activity is due to denaturation of the enzyme in dilute solution.

The usual method of activating aconitase consisted of incubating the enzyme for 1 hr. at 0° in the presence of 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine; a sample of the activated enzyme was then added to the test medium. (This method of activation will be referred to as method 1.) Thus when 0·1 ml. of the activated enzyme solution was added to 5 ml. of medium, the concentrations of Fe^{2+} and cysteine in the medium were reduced to 10^{-5}M and 2×10^{-4}M, respectively. When non-activated aconitase was added to a medium containing the lower concentrations of Fe^{2+} and cysteine and incubated for 5 min. at 30° before the addition of substrate, the enzyme showed no activity. When the concentrations of Fe^{2+} and cysteine in the medium were increased to 5×10^{-4}M and 10^{-2}M, respectively, the enzyme showed the same activity over the initial period of the reaction as it did after activation by method 1. (The activation of aconitase for 5 min. at 30° in the presence of 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine in the test medium will be referred to as method 2.) Fig. 2 shows the effect of adding the non-activated enzyme directly to a mixture of Fe^{2+}, cysteine and substrate. It will be noted that there is a distinct lag period of about 3 min. and that the reaction rate is slower than that of the control in which the enzyme was pre-incubated with the Fe^{2+} and cysteine before the addition of substrate. The fall in the control rate after the first 10 min. was a characteristic feature of the reaction after activation of the enzyme by method 2.

Activation of aconitase under strictly anaerobic conditions did not increase the enzyme activity, but when the enzyme–Fe^{2+}–cysteine mixture was shaken in air during the activation period, the enzyme showed no activity on the addition of substrate. The activated enzyme could also be inactivated by shaking in air during the course of the reaction; the degree of inactivation was dependent on the method of activation. Fig. 3 shows that there is a slight loss of enzyme activity when the medium is rapidly shaken in air after the addition of the enzyme activated by method 1. On the other hand, there is a marked loss of activity under these conditions when the enzyme is activated in the medium by method 2 before the addition of substrate. It would seem likely that the inactivation of aconitase is due to the absorption of the enzyme by suspended cystine formed as a result of the catalytic oxidation of cysteine by Fe^{2+} in presence of oxygen (Mathews & Walker, 1909). Although the addition of cystine to the medium in an amount less than that required for a saturated solution did not cause any inhibition, the above idea is consistent with the finding that the presence of high concentrations of cysteine which give rise to suspended cystine, caused more marked inhibition of aconitase. These results are of importance in so far as artificial systems for the oxidation of citrate are concerned. Activation of aconitase in such a way that the concentrations of Fe^{2+} and cysteine in the test medium are minimal is clearly preferable if aconitase is to act at a maximum rate for long periods of time under aerobic conditions.

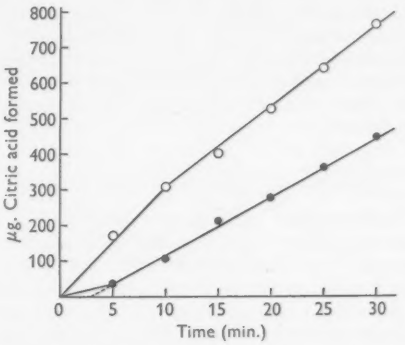

Fig. 2. The activation of aconitase by the addition of Fe^{2+} and cysteine to the test medium. 12 μg. of the non-activated aconitase preparation were added to the test medium which contained 5×10^{-4}M-Fe^{2+}, 10^{-2}M cysteine and 4×10^{-3}M *cis*-aconitate. Total volume 5·0 ml., temp. 30°, no buffer, pH 7·4. ○, aconitase incubated 5 min. with Fe^{2+} and cysteine before the addition of substrate; ●, aconitase added to a mixture of Fe^{2+}, cysteine and substrate without pre-incubation.

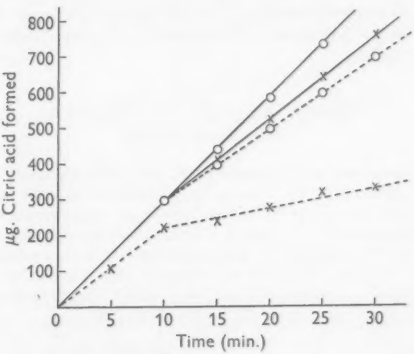

Fig. 3. The effect of rapid shaking in air on the activity of activated aconitase. The amount of aconitase added was equivalent to 12 μg. of the final aconitase preparation. Temp. 30°, no buffer, pH 7·4. ○, aconitase activated by method 1 as described in the text; ×, aconitase activated by method 2 as described in the text; ——, non-shaken; ----, shaken.

Specificity of ferrous ion

There was no relationship between the ability of a metal to oxidize cysteine catalytically and the ability of the system to activate aconitase. It was found that Cu^{2+} and Mn^{2+} at concentrations of 5×10^{-4}M did not bring about any activation of aconitase. Moreover, when this concentration of Cu^{2+} was added to the usual activation system containing Fe^{2+} and cysteine, the activity of the enzyme was inhibited by 50%. Krebs & Eggleston (1944) also found that aconitase was inhibited by Cu^{2+}. It is of interest that Cu^{2+} and Mn^{2+}, which belong to the same transition series as Fe^{2+} and which therefore have similar properties, do not activate aconitase.

Activation of aconitase by reducing agents

Table 2 shows the relative effectiveness of four reducing agents in activating aconitase in the presence of a fixed amount of Fe^{2+}. At a level of 0·01 M, cysteine brings about the greatest activation of aconitase, whilst thioglycollate, ascorbic acid and glutathione are decreasingly effective in the given order. When the concentration of reducing agent was increased to 0·025 M, the degree of activation was increased in all cases, being most marked with glutathione. Cysteine is still the most effective, but the activation by glutathione is increased to such an extent that it is as effective as thioglycollate. The higher concentration of ascorbic acid caused only a small increase in the enzyme activity, so that at this level it was the least effective. It was found that when the concentration of cysteine and ascorbic acid was increased to 0·1 M, the enzymic activity was slightly less than that obtained at a concentration of 0·01 M.

Table 2. *Effect of reducing agents on aconitase activity*

Aconitase was incubated with the reducing agent and 5×10^{-4}M-Fe^{2+} for 1 hr. at 0°. Samples (0·25 ml.), equivalent to 15 μg. of the final aconitase preparation, were added to media containing 1·5 ml. of 0·1 M phosphate buffer (pH 7·7) and 1·25 ml. of *cis*-aconitate (final concentration $6{\cdot}7 \times 10^{-3}$M). Reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citrate. Temp. 22°.

Reducing agent	Aconitase activity (μg. citrate formed) at concentration of reducing agent of 0·01 M	0·025 M
Cysteine	178	265
Ascorbic acid	100	114
Glutathione	23	182
Thioglycollate	129	182

Role of ferrous ion in the action of aconitase

In order to elucidate the role played by Fe^{2+} in the action of aconitase, a quantitative study was made of the effect of the Fe^{2+} concentration on the activity of aconitase. The observations on the relationship between the Fe^{2+} concentration and the reaction velocity in the presence of cysteine and ascorbic acid, as shown in Fig. 4, conform to a simple Michaelis–Menten relationship, i.e. to the equation $v = Vx/(K_x + x)$, where v = initial velocity of the reaction at a standard concentration of substrate in the presence of a reducing agent, V = maximum reaction velocity, x = concentration of Fe^{2+}, and K_x = dissociation constant of the Fe^{2+}–enzyme complex formed according to the equation

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{–enzyme}.$$

These results are consistent with the hypothesis that Fe^{2+} combines with aconitase in the ratio of one ferrous ion to one active centre to form the Fe^{2+}–enzyme complex, which is at least partially responsible for enzyme activity.

This interpretation is valid only because it was known from previous experiments that the above equilibrium was established during the preincubation of the enzyme with Fe^{2+} and the reducing agent for 1 hr. at 0°. The substrate was not added and the reaction rate was not determined until

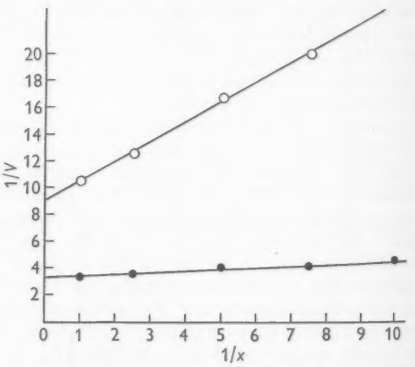

Fig. 4. The effect of Fe^{2+} concentration on aconitase activity. The graphs are plotted according to the method of Lineweaver & Burk (1934). Aconitase was activated in the presence of 10^{-2}M cysteine or ascorbic acid and various concentrations of Fe^{2+} at pH 7·4 for 1 hr. at 0°. Samples (0·25 ml.) of the enzyme solutions, containing 15 μg. of the final aconitase preparation, were added to media containing 1·5 ml. of 0·1 M phosphate buffer (pH 7·7) and 1·25 ml. of neutralized *cis*-aconitate (final concentration $6{\cdot}7 \times 10^{-3}$M). The reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the solutions analysed for citrate. Temp. 22°. x, concentration of $Fe^{2+} \times 10^4$M; V, μg. of citrate formed/15 min. $\times 10^{-3}$; ●, cysteine; ○, ascorbic acid.

after the equilibrium was established, so it can be taken that that reaction rate was proportional to the amount of the Fe^{2+}–enzyme complex formed. As the results conform to a Michaelis–Menten relation, the reaction between Fe^{2+} and the reducing agents (see p. 690) apparently does not effectively lower the Fe^{2+} concentration.

As the final enzyme preparation was subjected to prolonged dialysis against citrate buffer (pH 5·7) before use, it was assumed that there was little or no Fe^{2+} remaining in the preparation. It was on this basis that the dissociation constants were calculated to be $3{\cdot}9 \times 10^{-6}$ M in the presence of cysteine and $1{\cdot}7 \times 10^{-5}$ M in the presence of ascorbic acid. The above values may not be precise in view of the fact that the enzyme did possess slight residual activity in the absence of both Fe^{2+} and a reducing agent (see Fig. 1). The values for the dissociation constants may vary from one preparation to another. However, it is clear that aconitase has a very high affinity for Fe^{2+}. It can also be seen in Fig. 4 that the absolute value of the maximum rate in the presence of cysteine is over twice as great as that obtained in the presence of ascorbic acid.

The relationship between the enzyme activity and the concentration of Fe^{2+} in the presence of thioglycollate was also determined. In this case, the enzyme activity fell off so sharply that it was not possible to determine accurately the reaction velocities under the same conditions as used for cysteine and ascorbic acid. The enzyme activity at a Fe^{2+} concentration of 5×10^{-5} M was only about 8% of the activity at a Fe^{2+} concentration of 10^{-4} M.

Role of reducing agents on the action of aconitase

The above experiments indicate that reducing agents as well as Fe^{2+} are concerned in the formation of the active aconitase complex. Therefore, an investigation was made of the effect of varying concentrations of reducing agents on the aconitase activity in the presence of 5×10^{-4} M-Fe^{2+}.

Cysteine, thioglycollate and ascorbic acid. The observations on the relationship between the concentration of cysteine, thioglycollate and ascorbic acid and the reaction velocity, as shown in Fig. 5, conform to a simple Michaelis–Menten relation over the concentrations used, i.e. to the equation $v = Vc/(K_c + c)$, where v = initial velocity of the reaction at a standard concentration of substrate, in the presence of 5×10^{-4} M-Fe^{2+}; V = maximum reaction velocity; c = concentration of the reducing agent, and K_c = dissociation constant of the reducing agent–enzyme complex, formed according to the equation

$$\text{enzyme} + \text{reducing agent} \rightleftharpoons \text{enzyme–reducing agent}.$$

It should be pointed out that the results obtained with cysteine and thioglycollate were somewhat variable. The points obtained in a number of experiments were scattered around, rather than lying directly on a straight line. The results illustrated in Fig. 5 are typical of a number of experiments where the points fell on a straight line. In all experiments the values obtained for the reciprocals of the reaction velocities at each concentration of reducing agent were within +10% and −5% of the values shown. At lower concentrations of these reducing agents (below 2×10^{-3} M), the points obtained fell well above the straight line passing through the points obtained with higher concentrations. It would seem as though these variations could be related to the catalytic oxidation of cysteine and thioglycollate by Fe^{2+} in the presence of dissolved oxygen. With low concentrations of these substances the relative reduction in the concentration would be marked. On the other hand, with ascorbic acid the points obtained at all concentrations invariably fell on a straight line. This substance is not catalytically oxidized by Fe^{2+}. However, it was clear that the activity of aconitase is dependent on the concentration and nature of the reducing agent. The results can be interpreted to indicate that each reducing agent combines with aconitase in the presence of Fe^{2+} in the ratio of one molecule of the reducing agent to one active centre of the enzyme to form an enzyme–reducing agent

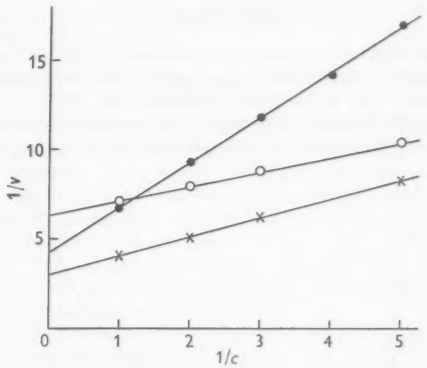

Fig. 5. The effect of cysteine, thioglycollate and ascorbic acid concentrations on aconitase activity in the presence of Fe^{2+}. The graphs are plotted according to the method of Lineweaver & Burk (1934). Aconitase (60 μg. of the final preparation/ml.) was incubated with 5×10^{-4} M-Fe^{2+} and various concentrations of the reducing agents for 1 hr. at 0° and pH 7·4. The same enzyme solution was used in all experiments. The enzyme activity was determined as described in Fig. 4. c, concentration of reducing agent $\times 10^2$ M; v, μg. of citrate formed/15 min. $\times 10^{-3}$; ×, cysteine; ○, ascorbic acid; ●, thioglycollate.

complex. No activities were obtained unless Fe^{2+} was also present. Again this interpretation is valid only because the system had attained equilibrium before the reaction rates were determined.

The dissociation constants of the enzyme–reducing agent complexes were calculated to be $2{\cdot}3-3{\cdot}6\times10^{-3}$M for cysteine, $4{-}6\times10^{-3}$M for thioglycollate and $1{\cdot}2\times10^{-3}$M for ascorbic acid. These values indicate that the affinity of the enzyme for ascorbic acid is greater than for cysteine, which in turn is greater than that for thioglycollate. There is no relationship between the affinities of the enzyme for the reducing agent and the maximum activity of the enzyme in the presence of the same reducing agents. Cysteine gives the greatest maximum velocity, ascorbic acid the lowest, whilst thioglycollate gives an intermediate value.

Glutathione. When glutathione was tested for its ability to activate aconitase, it was seen (Table 2) that very little activation was obtained at a concentration of 0·01M. However, an appreciable activation of the enzyme was obtained with 0·025M glutathione. A study of the effect of glutathione concentration on the enzyme activity, as shown in Fig. 6, indicates that over the range of concentrations required for enzyme activity there is no simple Michaelis–Menten relation as obtained with cysteine, thioglycollate and ascorbic acid. Thus the mechanism of the activation must differ in some respect from that obtained with the other reducing agents.

DISCUSSION

The marked activation of aconitase by Fe^{2+} in the presence of a reducing agent and the failure of other metal ions to activate, indicate that Fe^{2+} is a specific integral component of the aconitase system. The observations on the relationship between the

Fig. 6. The effect of glutathione concentration on aconitase activity. The graph is plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 5. *S*, concentration of glutathione × 10M, and *v*, μg. of citrate formed/15 min. × 10^{-3}.

Fe^{2+} concentration and the enzyme activity are consistent with the idea that Fe^{2+} reacts with the enzyme in the ratio of one ferrous ion to one active centre of the enzyme. Thus the Fe^{2+}–enzyme complex can be considered as an active component of the system. If this complex only were concerned in the activation, then when the Fe^{2+} concentration rises sufficiently the limiting concentration of the Fe^{2+}–enzyme complex should be independent of the nature of the reducing agent. As the activation of the enzyme is further increased by the presence of a reducing agent and influenced by the type of reducing agent, this cannot be the only reaction concerned in the activation. This idea is strengthened by the finding that the dissociation constant of the Fe^{2+}–enzyme complex varies according to the reducing agent present.

The function of the reducing agents is not likely to be related to their ability to maintain the iron in the ferrous state as suggested by Dickman & Cloutier (1951). The concentration of the reducing agents was always in excess of that of Fe^{2+} and the oxidation–reduction potentials are such that they favour the reduction of iron, so only a small amount of Fe^{3+} must be present under the conditions of these experiments. Therefore, it is unlikely that a Michaelis–Menten relationship between the concentration of the reducing agents and the enzyme activity would be found if this were the sole function of the reducing agents. Moreover, if this were the case, the maximum velocity of the reaction should be independent of the nature of the reducing agent. It is also unlikely that the activation of aconitase by the reducing agents is concerned with the reduction of disulphide linkages on the surface of the enzyme (Dickman & Cloutier, 1951). The Michaelis–Menten relations indicate that only one molecule of reducing agent reacts with each active centre, and preliminary studies with arsenical inhibitors indicate that aconitase is a monothiol enzyme (unpublished work). If this is the case and provided that there was no steric hindrance, the active monothiol enzyme molecules could give rise to inactive dimers as a result of the formation of disulphide linkages. However, if the reducing agents were concerned in splitting disulphide linkages, it would be expected that the maximum enzyme activity would be independent of the nature of the reducing agent.

It seems as though the results with cysteine, thioglycollate and ascorbic acid can best be interpreted by considering that they react with aconitase in the ratio of one molecule of the reducing agent to each active centre of the enzyme. The reducing agent can then be considered as taking part in the formation of the active enzyme complex.

The situation is that one molecule of reducing agent and one ferrous ion react with aconitase to form the active complex. Schubert (1932) showed

that cysteine and thioglycollate react with Fe^{2+} to form a ferrous–biscysteine or a ferrous–bisthioglycollate complex, and possibly ascorbic acid can form the same type of complex. Therefore, it seems as though Fe^{2+} might react with aconitase and that the Fe^{2+}–enzyme reacts in turn with one molecule of reducing agent to form the active complex. The nature of this complex would be determined by the reducing agent. The results clearly show that the equilibria

$$\text{enzyme} + Fe^{2+} \rightleftarrows Fe^{2+}\text{–enzyme}$$

and

$$\text{enzyme} + \text{reducing agent} \rightleftarrows \text{enzyme–reducing agent}$$

are not independent.

A similar pattern for the activation of arginase by Fe^{2+}–cysteine and Fe^{2+}–ascorbic acid was reported by Purr & Weil (1934). These authors concluded that the activation mechanism is probably connected with a specific oxidation–reduction potential. However, the activation of this enzyme must differ from that of aconitase in so far as activation can also be brought about by other metallic ions alone, e.g. Co^{2+} and Mn^{2+} (Mohamed & Greenberg, 1945). With aconitase, it remains to be determined whether or not the organic activator must be a reducing agent and/or a compound capable of forming a complex with Fe^{2+}. Until this point is decided, it may be better to consider the active form of aconitase as being an enzyme–Fe^{2+}–activator complex.

Since the substrates of aconitase are also capable of forming complexes with Fe^{2+} (Dickman & Cloutier, 1951) it is tempting to suggest that the remaining free valencies of Fe^{2+} are concerned in linking the substrate to the enzyme. On the other hand, if Fe^{2+} alone were concerned with linking the substrate to the enzyme and the reducing agent were capable of reacting with the Fe^{2+} bound to the enzyme, there would be in effect competition between the activators of the enzyme and the substrates. At the same time, it must be borne in mind that the Fe^{2+} and reducing agents could function by fixing the degrees of freedom of the enzyme, so that the substrate could be linked to some other part of the enzyme molecule.

The results with glutathione do not completely fit in with the above hypothesis. Glutathione does activate aconitase, but if an enzyme–Fe^{2+}–glutathione complex is required for activity, the glutathione dissociation differs from that with the other activators. Moreover, the concentration of glutathione required for maximum activation is very much higher than that required with the other reducing agents. It is interesting to compare this result with those obtained by Kubowitz (1935) on the reaction between Fe^{2+} and glutathione. He found that it was possible to form a carbon monoxide complex of ferrous glutathione only in the presence of high concentrations of glutathione and concluded that this was due to the high dissociability of the ferrous–glutathione complex. With a Fe^{2+} concentration of 2×10^{-3} M, he found that the concentration of glutathione required to form the carbon monoxide complex of ferrous glutathione was 10^{-1} M. On this basis, a Fe^{2+} concentration of 5×10^{-4} M would require a glutathione concentration of $2{\cdot}5 \times 10^{-2}$ M to form the complex. This is in fact the concentration of glutathione found to be required for maximum activation of aconitase. It seems to follow from these results that the activation of aconitase by glutathione is related to its ability to form a Fe^{2+} complex.

The problem of the activation of aconitase is indeed a complex one. Although the reducing agents increase the activity of the enzyme above that obtained with Fe^{2+} alone, it is clear that they are not essential for aconitase activity; the addition of Fe^{2+} alone greatly enhances the activity of the purified enzyme. The complexities of the equilibria between the Fe^{2+} and the enzyme, activators and substrates of aconitase further complicate the problem and make the kinetics of the system difficult. Perhaps further light may be thrown on the problem by the determination of the Michaelis constants and maximum velocities of aconitase for its three substrates in the presence of different organic activators. It would also be of interest to determine whether or not Fe^{2+} can form mixed complexes with each of the activators and the substrates of aconitase.

SUMMARY

1. It was shown that the final purified aconitase preparation possessed little activity in the absence of cofactors. It was not activated by cysteine alone; the addition of Fe^{2+} increased the activity 15-fold, whilst the addition of Fe^{2+} and cysteine gave a 70-fold increase in activity.

2. The optimum conditions required both for activating aconitase and for the estimation of enzymic activity were determined.

3. Aconitase was not activated by Mn^{2+}, and Cu^{2+} was inhibitory.

4. A Michaelis–Menten relationship was shown to exist between the concentration of Fe^{2+} and the enzyme activity. It was concluded that a Fe^{2+}–enzyme complex was partly responsible for aconitase activity.

5. A Michaelis–Menten relationship was also found to exist between the enzyme activity and the concentration of the reducing agents cysteine, thioglycollate and ascorbic acid. It was concluded that these compounds form an enzyme–reducing agent complex which is partly responsible for enzyme

activity. Only higher concentrations of glutathione appreciably activated aconitase; there was no simple Michaelis–Menten relationship between the concentration of glutathione and the enzyme activity.

6. The conclusion was drawn that the active form of aconitase is either an enzyme–Fe^{2+}–reducing agent or an enzyme–Fe^{2+}–activator complex.

It is a pleasure to express my thanks to Sir Rudolph Peters, F.R.S., Dr R. B. Fisher and Dr R. Cecil for their interest and advice during this work. Thanks are also due to Miss S. Read for skilled technical assistance.

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The Relationship of Quaternary Ammonium Salts to the Anionic Sites of True and Pseudo Cholinesterase

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(*Received* 10 *May* 1954)

In recent experiments on serum cholinesterase (Bergmann & Wurzel, 1954) evidence for the presence of an anionic site near the esteratic site of the enzyme was obtained and the conclusion was reached that the active surface of this enzyme is essentially similar to that of true cholinesterase. Since the differences between these two esterases have in the past been ascribed to the absence or unimportance of a negative site in the pseudo enzyme (Adams & Whittaker, 1950), it now becomes necessary to explain on a new basis the specific substrate affinity to either enzyme.

Paton & Zaimis (1949), in their study on alkane bistrimethylammonium salts ('methonium' compounds), reported only little inhibitory activity towards serum cholinesterase, but pronounced activity against laked erythrocytes of rabbits. The results of Bergmann & Wurzel (1954), obtained under different experimental conditions, were not in accord with their findings and indicated that the ratios of affinities of inhibitors of the methonium series towards the two enzymes reverse with increasing chain length. It was expected that a quantitative study of this problem would not only clarify the differences reported, but also contribute to a more precise definition of the active surface of the two cholinesterases and to an understanding of their physiological role. We have therefore investigated the inhibitory effect of two homologous series of ammonium derivatives, viz. monoacidic and diacidic quaternary ammonium bases with the nitrogen attached to the ends of unbranched paraffin chains, in order to evaluate quantitatively the factors determining the intermolecular forces between these enzymes and their inhibitors.

MATERIALS AND METHODS

Substrates. Commercial acetylcholine bromide (ACh) was used. Benzoylcholine chloride (BCh) was obtained through the courtesy of Dr Aeschlimann, Hoffmann–La Roche, Nutley, New Jersey. Diacetin was supplied by British Industrial Solvents Ltd., London.

Inhibitors. Butane-1:4-bistrimethylammonium dibromide ('tetramethonium') and heptamethonium were a gift of Dr H. J. Barber of May and Baker Ltd., Dagenham, Essex, and decamethonium of Messrs Allen and Hanburys Ltd., Manchester, England. All other methonium compounds were synthesized in our laboratory by a method to be published elsewhere. Monoacidic quaternary ammonium salts were prepared from trimethylamine and alkyl halides in dilute ethanolic solutions. The lower members, methyl to *n*-pentyl, were crystallized as bromides. However, the halides of higher members of the series proved to be too hygroscopic for purification. It was, therefore, necessary to use the perchlorates, the properties of which are summarized in Table 1.

Enzyme preparations. Purified, crystalline cholinesterase of human plasma (fraction IV-6-3) was obtained through the courtesy of Dr Ruth M. Flynn, Department of Biochemistry, Harvard Medical School (a brief description of the preparation of this material is given by Edsall, 1947). This material, in a dilution of 1:10000 and at 37°, hydrolyses 9 μmoles/ml./hr., when ACh, 6×10^{-2}M, is used as substrate. Cholinesterase from the electric organ of *Electrophorus electricus* ('eel esterase') was purified according to Rothenberg & Nachmansohn (1947). The solution so obtained, in a dilution of 1:1000, hydrolyses at 23° 5 μmoles/ml./hr., when ACh, 4×10^{-3}M, serves as substrate. Activities were measured under the conditions given in the following paragraph.

The enzyme solution was incubated with the inhibitor for 45 min., before the substrate was added, and the hydrolytic activity was measured manometrically. The buffer used as medium for these experiments contained 0·1M-NaCl, 0·026M-$NaHCO_3$ and 0·04M-$MgCl_2$. The pH was adjusted to 7·3. The gas phase consisted of 95% air and 5% CO_2. The I_{50} values, i.e. the concentrations of inhibitors producing 50% inhibition, were obtained graphically by plotting on semilogarithmic paper the percentage inhibition as a function of inhibitor concentration.

RESULTS

In Table 2 the inhibitory activity of compounds ranging from tetra- to dodeca-methonium is compared. The values for penta- and hexa-methonium against eel esterase were determined with crystalline salts, whereas earlier experiments (Bergmann & Wurzel, 1954), had been made with commercial preparations (Allen and Hanburys Ltd.). The I_{50} value of any reversible, competitive inhibitor depends on the substrate concentration according to equation 1, namely

$$\frac{v_0}{v_i} = 1 + \frac{I \times K_m}{K_i(K_m + S)}, \qquad (1)$$

Table 1. *Properties and analyses of alkyltrimethylammonium perchlorates*

Analyses were performed according to the method of Bodenheimer & Weiler, details of which will be described elsewhere. However, the conditions are as follows: 0·5–1·0 mg. of the organic perchlorate is dissolved in water and added to a solution of copper–pyridine–nitrate complex of known concentration. After the copper–pyridine–perchlorate has precipitated completely (during 12 hr. standing), the supernatant is removed and its extinction compared with that of the original reagent solution. The difference permits the evaluation of the perchlorate concentration in the sample by comparison with a calibration curve, obtained with analytically pure potassium perchlorate.

Alkyl group	M.p. (°)	Solvent for recrystallization	Crystal form	% ClO_4^- Calc.	% ClO_4^- Found
Hexyl	153	*n*-Propanol	Prisms	40·7	40·5
Heptyl	126	Ethyl acetate	Prismatic columns	38·5	38·6
Octyl	118	Ethanol	Plates	36·5	36·5

Table 2. I_{50} *values for monoacidic and diacidic quaternary ammonium compounds*

All salts are derived from trimethylamine and vary only in the length of the carbon chain of the fourth substituent, which is given in the first column by *n*, the number of carbon atoms in the chain. All experiments were carried out at 37°, apart from the system true cholinesterase–monoacidic bases, which was examined at 23°.

	Serum cholinesterase			
	Acetylcholine		Benzoylcholine,	True cholinesterase, acetylcholine,
n	6×10^{-2}M I_{50} (M)	4×10^{-3}M I_{50} (M)	4×10^{-3}M I_{50} (M)	4×10^{-3}M I_{50} (M)
Diacidic bases (methonium compounds)				
4	$4{\cdot}7 \times 10^{-2}$	$1{\cdot}8 \times 10^{-3}$	—	$8{\cdot}6 \times 10^{-2}$
5	$2{\cdot}1 \times 10^{-2}$	$2{\cdot}5 \times 10^{-3}$	—	$4{\cdot}8 \times 10^{-2}$
6	$1{\cdot}1 \times 10^{-2}$	$9{\cdot}7 \times 10^{-4}$	$7{\cdot}3 \times 10^{-3}$	$1{\cdot}9 \times 10^{-2}$
7	$3{\cdot}4 \times 10^{-3}$	$4{\cdot}0 \times 10^{-4}$	—	$1{\cdot}2 \times 10^{-2}$
8	$1{\cdot}9 \times 10^{-3}$	$3{\cdot}0 \times 10^{-4}$	$1{\cdot}7 \times 10^{-3}$	$2{\cdot}4 \times 10^{-3}$
9	$6{\cdot}2 \times 10^{-4}$	$1{\cdot}0 \times 10^{-4}$	$1{\cdot}0 \times 10^{-3}$	$3{\cdot}0 \times 10^{-4}$
10	$3{\cdot}5 \times 10^{-4}$	$4{\cdot}6 \times 10^{-5}$	$7{\cdot}0 \times 10^{-4}$	$2{\cdot}0 \times 10^{-5}$
12	$1{\cdot}2 \times 10^{-4}$	—	—	$8{\cdot}2 \times 10^{-6}$
Monoacidic bases				
1	—	—	—	$1{\cdot}5 \times 10^{-2}$
2	$1{\cdot}9 \times 10^{-1}$	—	—	$6{\cdot}8 \times 10^{-2}$
3	$1{\cdot}4 \times 10^{-1}$	—	—	$3{\cdot}0 \times 10^{-3}$
4	$5{\cdot}4 \times 10^{-2}$	—	—	$2{\cdot}0 \times 10^{-3}$
5	$1{\cdot}7 \times 10^{-2}$	—	—	$1{\cdot}5 \times 10^{-3}$
6	$1{\cdot}6 \times 10^{-2}$	—	—	$6{\cdot}9 \times 10^{-4}$
7	$4{\cdot}3 \times 10^{-3}$	—	—	$6{\cdot}0 \times 10^{-4}$
8	$2{\cdot}5 \times 10^{-3}$	—	—	—

where v_0 and v_i denote the rate of enzymic hydrolysis in the absence and presence of inhibitor respectively, S and I the molar concentration of substrate and inhibitor, K_m the Michaelis–Menten constant and K_i the equilibrium constant for the enzyme–inhibitor complex. Therefore figures for two different values of S are included in Table 2.

When pI_{50} ($-\log_{10} I_{50}$), was plotted as a function of n (= the number of carbon atoms separating the charged end-groups), a straight line was obtained for serum cholinesterase, whereas an S-shaped curve characterizes the eel esterase (Fig. 1). A simple law must therefore apply to the system serum-cholinesterase–methonium compounds, which can be formulated as follows: if in equation 1 we set $I = I_{50}$, i.e. $v_0 = 2v_i$, we obtain the relationship

$$K_i = I_{50}\,\frac{K_m}{K_m + S}, \qquad (2)$$

and thus for a given constant substrate concentration

$$\frac{{}^{n}I_{50}}{{}^{n-1}I_{50}} = \frac{{}^{n}K_i}{{}^{n-1}K_i}. \qquad (3)$$

Therefore $\log K_i$, like $\log I_{50}$, should be a linear function of n, i.e. the difference between successive $\log K_i$ values is constant. We may now derive directly the following equation:

$$\begin{aligned} RT\,(\log {}^{n}K_i - \log {}^{n-1}K_i) &= RT(\log {}^{n}I_{50} - \log {}^{n-1}I_{50}) \\ &= \Delta F_{n-1} - \Delta F_n = \text{const.} \qquad (4) \end{aligned}$$

We are thus justified in calculating directly from the slope of the straight lines in Fig. 1 the increment in free energy change, $\Delta\Delta F$, accompanying the addition of one —CH_2— group to the central chain of a methonium compound. This magnitude measures the energy gained by transfer of one aliphatic carbon atom from its free state in solution to the state of adsorption on the protein surface. At 310° K we obtain a value of about 500 cal./—CH_2— group. The identical slope, obtained for serum cholinesterase at two different substrate concentrations, shows that $\Delta\Delta F$, as expected, is independent of S.

These observations demonstrate a simple relationship for the system serum-cholinesterase–methonium compound. All other forces between inhibitor and enzyme being kept constant within this homologous series, each additional carbon atom produces the same increase in affinity. Thus each —CH_2— group is held by identical forces. Therefore, the structure of the active surface of this enzyme must be relatively 'simple', i.e. no steric restrictions are imposed on unbranched paraffin chains, at least up to 10 carbon atoms.

The sigmoid curve, shown in Fig. 1 for the system true cholinesterase–methonium compounds, must be due to some factor which 'disturbs' the simple relationship observed with the plasma enzyme in such a way that the affinity of an additional carbon atom in the paraffin chain is at first smaller than in the case of serum cholinesterase, but beyond a certain value of n increases rapidly. If the 'unspecific' forces of attraction between a —CH_2— group and a protein molecule are assumed to be constant, the disturbance probably results from the ionic groups terminating the paraffin chain. Assuming for the time being that the presence of the second quaternary group might be responsible, it can be expected that monoacidic quaternary ammonium salts would not be subject to such an interference and therefore should give a linear plot of $\log K_i$ versus n for both enzymes. This was indeed confirmed by the experimental results shown in Fig. 2. The straight line for serum cholinesterase is almost identical with the corresponding curve in Fig. 1, indicating that the second ammonium group in a methonium compound does not materially contribute to the forces binding these inhibitors to pseudo cholinesterase. The curve for true cholinesterase in Fig. 2 represents measurements at 23° and from its slope $\Delta\Delta F$ is found to be about 300 cal./—CH_2— group.

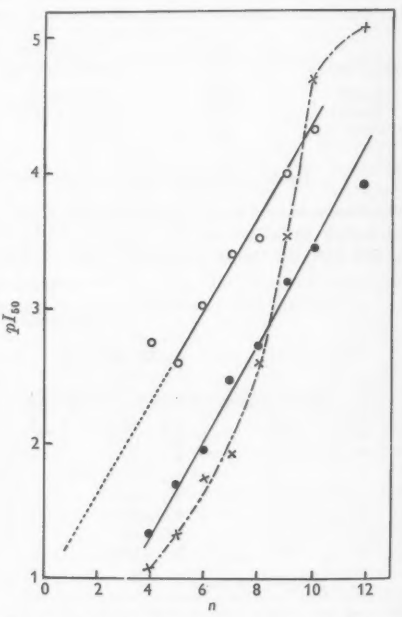

Fig. 1. pI_{50} values of methonium compounds as function of n, the number of carbon atoms between the terminal nitrogen atoms. ○—○, plasma cholinesterase at 37° + acetylcholine, 4×10^{-3} M; ●—●, same enzyme + acetylcholine, 6×10^{-2} M; ×—×, eel esterase at 37° + acetylcholine, 4×10^{-3} M.

Extrapolation of the straight lines in Figs. 1 and 2 to $n=1$ gives a 'theoretical' value of $^1I_{50}$, from which ^1K_i can be calculated using equation (2), if K_m is known. It should be noted that the value for tetramethylammonium, derived in this way, differs from the experimental figure. However, for small values of n, the interaction between an aliphatic carbon and the protein is probably influenced by the neighbouring electrically charged groups. The Michaelis–Menten constant for the system serum cholinesterase-ACh was determined by the method of Dixon (1953). For 'true' cholinesterase, instead of K_m the value of K_1 (Wilson & Bergmann, 1950*b*) was used, although it has been shown in the meantime that the derivation of this constant was based on the incorrect assumption that the anionic site does not undergo any change within the experimental pH range (Bergmann & Shimoni, 1952). Since in tetramethylammonium the aliphatic 'side chain' has been eliminated, ^1K_i measures the equilibrium for the electrostatic combination of this ion with the anionic site. According to Hammett (1940) the coulombic energy can be calculated from equation 5

$$\Delta F_1 = -RT \log {}^1K_i = \frac{e_1 \times e_2}{D \times r}, \qquad (5)$$

where e_1 and e_2 refer to the charge of inhibitor and enzyme respectively. For the dielectric constant D we use the value 30, as proposed by Pressman, Grossberg, Pence & Pauling (1946). Since $e_1=1$, we can now derive values for the interionic distance r, when $e_2=1$, 2, etc. As shown in Table 3, we obtain with serum cholinesterase $r=5{\cdot}3$ Å, for $e_2=1$ and 10·6 for $e_2=2$. Since the radius of the tetramethylammonium ion is about 4 Å and the C—O bond length of a carboxylate ion 1·4 Å, the distance of closest approach must be of the order of 5·5 Å. For true cholinesterase we find $r=2{\cdot}75$ Å if $e_2=1$. This value evidently is too small. The correct distance $r=5{\cdot}5$ Å is obtained for $e_2=2$. This result suggests not only that two negative sites must be present in the neighbourhood of the esteratic site of true cholinesterase, but also that they must be located close to each other so as to enable them to exert their attractive force on a single positive ion simultaneously.

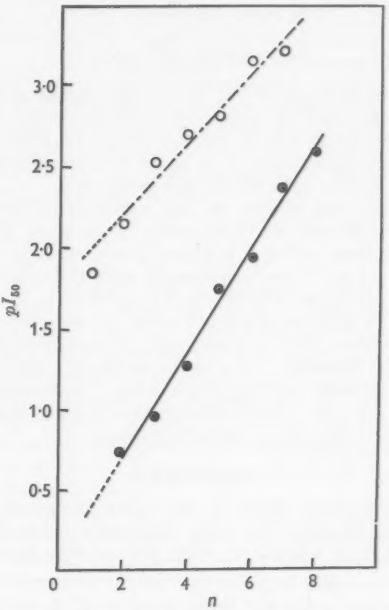

Fig. 2. pI_{50} values of n-alkyltrimethylammonium salts as function of n, the number of carbon atoms in the n-alkyl group: ○, eel esterase at 23° + acetylcholine, 4×10^{-3} M; ●, plasma cholinesterase at 37° + acetylcholine, 6×10^{-2} M.

Table 3. *Free energies, interionic distances and number of anionic sites*

The calculations for this table are based on the formula

$$\Delta F_{\text{molar}} = \frac{Ne_1 \times e_2}{D \times r} = -2{\cdot}3\,RT \log K,$$

where ΔF denotes the free energy change of the process under consideration and K its equilibrium constant; N = Avogadro's number = $6{\cdot}03 \times 10^{23}$; e_2 = the electronic charge of the active surface of the enzyme and e_1 = the charge of substrate or inhibitor respectively. These charges are expressed as multiples of $4{\cdot}8 \times 10^{-10}$ e.s.u. The dielectric constant D of the medium is taken as 30 according to Pressman *et al.* (1946); r, the distance of closest approach of the oppositely charged particles, is obtained in cm., but shown after conversion to Å.

	Constant used	ΔF molar (cal.)	$\frac{e_2}{4{\cdot}8 \times 10^{-10}}$	r(Å)
'Pseudo' cholinesterase	$K_m = 5{\cdot}56 \times 10^{-3}$	3200	—	—
	$^1K_i = 3{\cdot}6 \times 10^{-2}$*	2030	1	5·3
			2	10·6
'True' cholinesterase	$K_1 = 2{\cdot}6 \times 10^{-4}$†	4850	—	—
	$K_2 = 3{\cdot}2 \times 10^{-2}$†	2020	—	—
	$^1K_i = 6{\cdot}3 \times 10^{-4}$*	4350	1	2·75
			2	5·5

* Extrapolated value for tetramethylammonium.
† Taken from the paper of Wilson & Bergmann (1950*b*). See Discussion.

If this conclusion is correct, then monoacidic quaternary ammonium bases produce effective inhibition of true cholinesterase already when only one inhibitor molecule is bound by the enzyme. The number n in the enzyme–inhibitor complex EI_n can be measured in the following way: if in equation 1 we replace I by I^n, suitable transformation leads to expression 6

$$\log (v_0/v_i - 1) = n \log I + C, \qquad (6)$$

where $$C = \log \frac{K_m}{K_i(K_m + S)}.$$

A plot of $\log (v_0/v_i - 1)$ versus $\log I$ should give a straight line, the slope of which yields n directly. As shown in Fig. 3, for the system eel esterase–tetramethylammonium n is about 1, regardless of whether the substrate is ACh or diacetin.

Since our results on the inhibition of serum cholinesterase by methonium compounds differ from those of Paton & Zaimis (1949), we have also carried out a few experiments with the substrate used by these authors, viz. benzoylcholine. The I_{50} values as shown in Table 2 reveal the same trend as is observed for the corresponding concentration of ACh. However, owing to the smaller K_m value for the system benzoylcholine–serum cholinesterase, i.e. $2{\cdot}5 \times 10^{-3}$, the inhibitory effect is much smaller than in the case of ACh as substrate.

DISCUSSION

The negative charge in the active surface of true cholinesterase has been previously determined. Adams & Whittaker (1950) derived their figure of

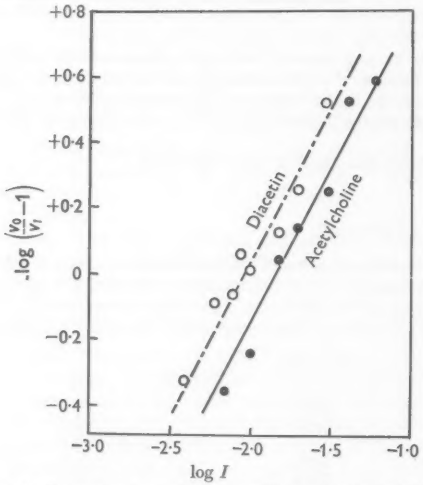

Fig. 3. Determination of the number of tetramethylammonium ions combining with the active surface of true cholinesterase, according to equation 6. Acetylcholine, 4×10^{-3} M; diacetin, $4{\cdot}3 \times 10^{-1}$ M.

$e_2 = 1$ from a comparison of the K_i values of choline for the two cholinesterases. The authors assumed $e_2 = 0$ for the pseudo enzyme and used the difference of $\log K_i^{\text{true}} - \log K_i^{\text{pseudo}}$ to eliminate all other 'unspecific' binding forces and to single out the coulombic energy of attraction for the negative site of true cholinesterase. In the light of our present results, however, they measured the charge difference between the two enzymes. Since we now have derived the value of $e_2 = 1$ for serum cholinesterase, the calculation of Adams & Whittaker leads logically to $e_2 = 2$ for the true enzyme.

It is more difficult to recognize the error underlying the determination of the negative charge in true cholinesterase by Wilson & Bergmann (1950*a*), who also found $e_2 = 1$. These authors compared dimethylaminoethyl acetate at pH 6, where the ester exists almost completely in the ammonium ion form, and at pH 10, where practically only the free base is present in solution. The coulombic energy of attraction was derived from the difference of the $\log K_m$ values at pH's 6 and 10, assuming that at any pH the rate of hydrolysis of the charged form of the ester is a constant fraction of the rate for ACh. It was believed that the protein affinity changes in exactly the same way for both substrates, and that the anionic site does not undergo any changes within the experimental range. The incorrectness of this assumption has been demonstrated subsequently (Bergmann & Shimoni, 1952).

In an earlier paper (Bergmann, Wilson & Nachmansohn, 1950) it was considered that the 'combination of tetramethylammonium with one anionic site should have practically no effect on the reaction velocity, since it essentially replaces normal enzyme molecules by others which have about half the substrate affinity. Therefore, such a type of inhibitor must combine with both anionic sites and form an EI_2 compound in order to prevent the enzymic reaction.' It is now recognized that the spatial arrangement of the two anionic sites in true cholinesterase enables them to combine simultaneously with one and the same inhibitor molecule. Therefore the EI complex is effective as such. However, formation of an EI_2 complex at sufficiently high inhibitor concentrations is thereby not excluded.

The essential difference between the two types of cholinesterase has now been established to lie in the number of negative charges in the neighbourhood of the esteratic site. Experiments, to be published soon, on the pH dependence of the hydrolysis of various substrates show that the esteratic sites of these enzymes are identical in all essential properties. The present results support the previous claim (Bergmann *et al.* 1950) that the formation of an ES_2 compound in the system true cholinesterase–ACh is possible only because of the presence of two negative charges. In addition, it is now

recognized that these charges can approach each other to such a degree as to interact simultaneously with a single quaternary ammonium ion. The complex of three charged particles thus formed is probably arranged in such a way that their centres lie on a straight line and that the potential energy is at its minimum (Fuoss & Kraus, 1933). It might be argued that this description of the spatial arrangement of two negative sites with one positive ion leads to the alternative possibility that one anionic site combines with two positive particles. However, in view of the great difference in size of a carboxylate and a tetramethylammonium ion, the former is much less able to accommodate two units of the latter.

The distance between the two negative charges, which are most probably represented by two carboxyl groups, is, however, not fixed and therefore two positive ions can also be accommodated. Thus, when an ES_2 compound is formed, the two ester molecules are bound each by one anionic site, but compete for the single esteratic site. The two equilibrium constants, which were determined previously (Wilson & Bergmann, 1950*b*), viz. K_1 for the formation of the ES and K_2 of the ES_2 complex, enable us to calculate the difference in binding energy involved in these two cases. It is seen from Table 3 that the binding energy for the second substrate molecule is less than half the value for the first one. In addition, the figure for $\Delta F_2 = 2020$ cal. is practically identical with the value derived from ${}^1K_i$ for serum cholinesterase, viz. 2030 cal., indicating that in the ES_2 complex each cationic ester molecule is bound to one anionic site only. In analogy, since for true cholinesterase we find ΔF (tetramethylammonium) = 4350 cal., it can be concluded that in the ES complex the single substrate molecule is bound simultaneously to both negative sites. This explains the much higher affinity of ACh to true than to pseudo cholinesterase in a qualitative way. However, a quantitative description of the active surfaces will become possible only when all their constants, including the dissociation constants of the anionic sites, have been determined accurately.

Interesting conclusions can be drawn from the I_{50} values of methonium compounds. The single negative charge of serum cholinesterase can accommodate only one positive group. The second cationic end-group does not participate in the binding of the inhibitor, as shown by the closely similar values for ions of equal chain length having one or two quaternary ammonium groups (cf. Figs. 1 and 2). Surprisingly, the second end-group does not even contribute appreciably to the sum of the van der Waals's forces. This is probably due to efficient competition of negative ions in the medium in the absence of a second negative site in the active surface.

True cholinesterase, on the other hand, can accommodate both cationic groups of a methonium compound. However, due to the spatial proximity of the anionic sites to each other, the inhibitor must be 'bent' in order to fit the active surface. 'Bending' becomes much easier, as the chain length increases, since with short chains considerable deviation from the normal bond angles would be involved. Therefore the short molecules resist this process of accommodation to the spatial requirements of the active surface, and show less affinity towards true than towards pseudo cholinesterase. Nevertheless, for true cholinesterase, the electrostatic attraction between the second pair of opposite charges interferes with the coulombic forces of the first pair and thus lowers the inhibitory effect of the shorter members of the methonium series (C_1–C_7) below the I_{50} values of the corresponding monoacidic quaternary ammonium salts.

The S-shaped log S/activity curve of serum cholinesterase can now be explained as due to the presence of a single anionic site, which permits combination with only one cationic ester molecule. The bell-shaped curve for true cholinesterase is related to the two negative charges in the active surface. However, substrates have been found which produce bell-shaped curves even in the absence of any electrostatic forces (Bergmann & Shimoni, 1953). This problem will be dealt with in a future publication.

In the application of these results to *in vivo* effects of methonium compounds we are handicapped by the lack of information on the composition and concentration of the physiological substrate of various cholinesterases. First, the possibility must be considered, that in tissues a mixture of choline esters is present, the composition of which may vary from place to place (Banister, Whittaker & Wijesundera, 1953). Likewise, no reliable information is as yet available on the concentration of substrate or substrates, which is in equilibrium with these enzymes *in vivo*. Therefore, only qualitative conclusions about pharmacological effects can be drawn from *in vitro* measurements. We may, however, predict that all members of the methonium series should be active against both types of cholinesterases in qualitatively the same way. This has been demonstrated by us, e.g. for hexamethonium, which under suitable experimental conditions exhibits a clear-cut blocking effect on the neuromuscular junction, similar to that of decamethonium (unpublished results). It is therefore concluded that the localization of a specific pharmacological effect upon application of 'therapeutic' doses of methonium compounds is due to quantitative differences in the affinities of these inhibitors to the different cholinesterases and thus follows the distribution of these enzymes in the tissues. On the

other hand, the ratio of activity of, for example, hexa- to deca-methonium on the cholinesterases of a given organ may serve as indication of the type of enzyme predominating in this organ. A simple method for the identification of different types of cholinesterases in various tissues can be based on these observations, as will be shown in a subsequent paper.

Application of the method developed here to the measurement of electric charges in the active centre of other enzymes is now being investigated.

SUMMARY

1. A plot of $\log I_{50}$ (I_{50} = concentration of inhibitor, producing 50% inhibition) versus n, the number of carbon atoms between the terminal nitrogens of alkane bistrimethylammonium salts, gives a straight line for serum cholinesterase and a sigmoid curve for eel esterase.

2. The same plot for n-alkyltrimethylammonium salts gives straight lines for both enzymes. The slope of these lines permits calculation of the free energy of the transfer of one —CH_2— group from the solution to the enzyme surface.

3. The extrapolated value of the inhibitor constant for tetramethylammonium makes possible the calculation of the energy of coulombic attraction between this ion and the negative charge in the active surface. From this magnitude the distance of the closest approach can be derived when the charge at the enzyme is 1, 2, 3, etc. unit charges.

4. In this way it is found that serum cholinesterase contains a single, and true cholinesterase two, anionic sites. The latter must be located near to each other and be sufficiently 'elastic' to allow simultaneous interaction with a single positive ion.

5. The possibility is discussed that one molecule of either acetylcholine or inhibitors can be attached simultaneously to both anionic sites of true cholinesterase, whereas when two molecules of substrate are bound to one enzymically active site, each ester molecule combines with one anionic site only.

6. An explanation is forwarded for the different substrate concentration/activity curves of the two cholinesterases.

The authors wish to thank Professor C. Chagas, Department of Physiology, University of Brazil, Rio de Janeiro, and Professor F. Feigl, for the gift of the electric organ of *Electrophorus electricus*. Our thanks are also due to Dr H. J. Barber, of May and Baker Ltd., Dagenham, Essex; to Dr Aeschlimann of Hofmann–La Roche, Nutley, N. J.; to Messrs Allen and Hanburys Ltd., Manchester, England, and to British Industrial Solvents Ltd., London, England, for the materials supplied. This paper forms part of a Ph.D. thesis, submitted by R. Segal to the Faculty of Science of the Hebrew University, Jerusalem.

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A Colorimetric Method for the Estimation of Acetoacetate

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(*Received* 9 *July* 1954)

The coupling of aromatic diazo compounds with compounds containing a reactive methylene group to form 'mixed' azo compounds (tautomeric with hydrazones) was recognized as a general reaction following Meyer's (1877) synthesis of phenylazoacetoacetic ester from benzenediazohydroxide and ethyl acetoacetate. Japp & Klingemann (1888) found that when equimolar amounts of acetoacetic acid and benzenediazohydroxide were coupled in acetate buffer, CO_2 was eliminated and methylglyoxal ω-phenylhydrazone was formed (eqn. 1, $R=C_6H_5$). Bamberger (1892) showed that, with excess benzenediazohydroxide in the presence of dilute alkali, a second or even a third diazo molecule coupled with acetoacetic acid, forming respectively *N*, *N'*-diphenyl-*C*-acetylformazan and phenylazo-(*N*, *N'*-diphenylformazan) (eqns. 2 and 3, $R=C_6H_5$).

$$CH_3.CO.CH_2.COOH + RN_2OH \rightarrow CH_3.CO.CH{:}NNHR + CO_2 + H_2O. \quad (1)$$

$$CH_3.CO.CH_2.COOH + 2RN_2OH \rightarrow CH_3.COC(N{=}NR)(N{-}NHR) + CO_2 + 2H_2O. \quad (2)$$

$$CH_3.CO.CH_2.COOH + 3RN_2OH \rightarrow RN{:}NC(N{=}NR)(N{-}NHR) + CH_3.COOH + CO_2 + 2H_2O. \quad (3)$$

A similar series of reactions between acetoacetic acid and *p*-nitrobenzenediazohydroxide has not been described. Rosenthal (1949) introduced a method for the estimation of acetoacetate in blood filtrates in which the products formed by coupling acetoacetate with diazotized *p*-nitroaniline were extracted into an immiscible solvent and estimated colorimetrically. The coupling reaction was carried out at an alkaline pH (unspecified) and the reaction products were not identified but were shown probably to be multiple.

In the present paper, conditions are defined under which even very small amounts of acetoacetate couple quantitatively with an excess of *p*-nitrobenzenediazohydroxide according to eqn. 2 above ($R=p\text{-}NO_2C_6H_4$). This reaction has been made the basis of a sensitive and specific method for the estimation of acetoacetate. The method has been applied to the estimation of acetoacetate formed by the oxidation of β-hydroxybutyrate by liver preparations and to its estimation in blood.

* J. G. Graves Fellow.

EXPERIMENTAL AND RESULTS

Materials

p-Nitroaniline (0·05%, *w/v*) *in* 0·05N-HCl. The *p*-nitroaniline was dissolved by boiling with water and the required amount of HCl. The solution was made up to the mark after cooling.

Diazo reagent. This was prepared immediately before use by adding 3·0 ml. $NaNO_2$ (0·5%, w/v; prepared daily) to 20·0 ml. *p*-nitroaniline solution. The colourless solution of the diazonium salt was chilled in an ice bath and 7·0 ml. 0·2M sodium acetate were added. Diazotization at room temperature rapidly gives an almost colourless solution of the comparatively stable diazonium salt of *p*-nitroaniline. Addition of sodium acetate brings the reagent to pH 4 and reduces its stability so that chilling is necessary to minimize spontaneous decomposition.

Acetoacetic acid. This was prepared by the complete hydrolysis of recently distilled ethyl acetoacetate according to the method of Ljunggren (1924). The removal of ethanol by vacuum distillation of the hydrolysate was not necessary since this compound did not interfere with the colour reaction for acetoacetate. After neutralizing the hydrolysate and making up to a convenient volume, the solution of acetoacetate was standardized manometrically according to the method of Edson (1935). The solution was stored at −18°. Restandardization after 30 days showed that about 5% of the acetoacetate had been lost through spontaneous decomposition. Dilutions of a freshly standardized solution of acetoacetate were used in preparing the calibration curves described below.

Oxaloacetic acid. The methods of Wohl & Oesterlin (1901) and Wohl & Claussner (1907) were used. It was dissolved and neutralized with NaOH immediately before use.

Mitochondrial preparation from rat liver. Freshly dissected chilled rat liver (5 g.) was ground for 2 min. with 0·25M sucrose solution (50 ml.) in a stainless-steel homogenizer. In this and all subsequent operations the temperature of the tissue was maintained at 0–5°. The ground liver was centrifuged twice at 500 *g* for 5 min., the sedimented nuclei, erythrocytes and intact cells being discarded. Mitochondria in the supernatant were sedimented by centrifuging for 20 min. at 2500 *g* and washed twice by resuspending in 50 ml. buffered saline (0·135M-KCl, 4 mM-Na_2HPO_4, 8 mM-$MgCl_2$ and 0·20M triethanolamine, pH 7·3) and centrifuging for 10 min. at the same speed. The final suspension was made in 50 ml. of the same buffered saline. For metabolic experiments 2·5 ml. of this suspension, containing approximately 10 mg. dry weight of tissue, and 1·0 ml. additions were present in each duplicate flask. Incubations were carried out at 20° in air using a Dubnoff Shaking Incubator (Precision Instruments Ltd., Chicago) or at 30° in standard Warburg vessels with oxygen in the gas space and alkali in the centre well.

Blood. Human blood was obtained by venepuncture and rat blood from the tail. The samples were heparinized, chilled in an ice bath and assayed within 2 hr. of collection.

Microanalyses. These were carried out by Drs Weiler and Strauss, Oxford.

Method

To 0·5 ml. of a neutral solution of acetoacetate in a glass-stoppered 10 ml. tube were added 0·5 ml. M sodium acetate–acetic acid buffer, pH 5·2, and 3·0 ml. freshly prepared diazo reagent. The final pH was 4·9–5·0. After standing for 30 min. at room temperature (about 22°) for colour development, the reaction was stopped by adding 1·0 ml. 5N-HCl and the product extracted by shaking with 4·0 ml. ethyl acetate (A.R.). For the reagent blank, water was used in place of acetoacetate solution.

The density of the ethyl acetate layer was read at 440 mμ. in a 1 cm. cell (Unicam SP. 600 spectrophotometer). This wavelength was selected on the basis of preliminary experiments and was used throughout the present work. From the absorption spectrum of the reaction product (Fig. 2) it can be seen that a wavelength of 450 mμ. is preferable and this is recommended for future work. The extracted colour was stable for at least 24 hr. Acidification with HCl stopped the coupling reaction, prevented further decomposition of the diazo reagent by restabilizing it as diazonium salt, and reduced the blank colour contributed by decomposition products such as *p*-nitrophenol. Extraction of the reaction product into ethyl acetate was complete, giving density readings inversely proportional to the volume of solvent used.

The almost-linear calibration curve obtained with solutions of acetoacetic acid containing 1–20 μg./ml. is shown in Fig. 1. The range of this calibration curve was extended to cover the direct assay of solutions of acetoacetic acid containing up to 100 μg./ml. by dilution of the final ethyl acetate extract to a convenient optical density. With larger amounts of acetoacetate, colour development became incomplete and preliminary dilution of the solutions was required.

An identical curve was obtained in similar experiments using solutions of acetoacetate in trichloroacetic acid, provided that the solutions were buffered so that the final pH for colour development was maintained at 4·9–5·0. With solutions containing, for example, 3% (w/v) trichloroacetic acid, 0·5 ml. M sodium acetate added in place of acetate buffer gave the required pH.

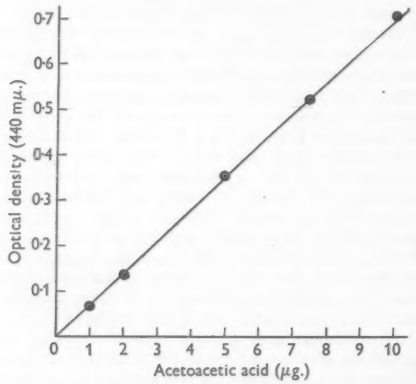

Fig. 1. Calibration curve for the estimation of acetoacetic acid.

Isolation and identification of the product of the coupling reaction

The product formed on coupling acetoacetate with diazotized *p*-nitroaniline under the conditions described above was insoluble. With small amounts of acetoacetate the reaction mixture became cloudy, with larger amounts a flocculent precipitate formed. In a large-scale experiment this precipitate was isolated, purified and shown to be N, N′-*bis*-(p-*nitrophenyl*)-C-*acetylformazan*. The coupling reaction therefore follows the course described by eqn. 2 ($R = p\text{-}NO_2C_6H_4$).

To 300 ml. of a neutral solution of acetoacetate (0·2 mg./ml.) were added 300 ml. M sodium acetate, pH 5·2, and 1800 ml. diazo reagent (molar ratio of *p*-nitroaniline to acetoacetate = 7:1). After stirring for 20 min. at room temperature the flocculent precipitate which formed was filtered off and dried *in vacuo* (wt. 203 mg., 97% of theoretical yield). It was recrystallized twice from ethanol in the form of small dark red-brown plates and dried at 50° *in vacuo*. The compound was insoluble in water, moderately soluble in acetone and very soluble in ethyl acetate and

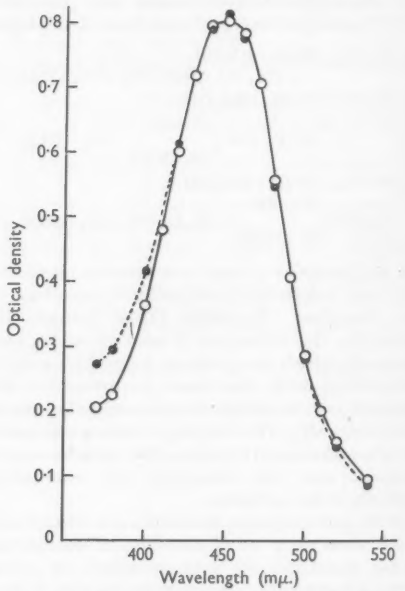

Fig. 2. Absorption spectra of *N*, *N*′-bis-(*p*-nitrophenyl)-*C*-acetylformazan and of the reaction product formed by coupling acetoacetate with diazotized *p*-nitroaniline. ○—○, *N*, *N*′-bis-(*p*-nitrophenyl)-*C*-acetylformazan, 1·00 mg. in 100 ml. ethyl acetate; ●- - -●, product from 11·5 μg. acetoacetate coupled under standard conditions (see text) and extracted into 4 ml. ethyl acetate.

benzene. It gave a violet colour with dilute NaOH and red-purple with concentrated H_2SO_4. It had m.p. 217–219° (uncorr.) with decomposition (rate of heating = 10°/min.) and ϵ 440 mμ. = $2{\cdot}85 \times 10^4$ in ethyl acetate at 22° (average of four experiments, range $2{\cdot}83$–$2{\cdot}86 \times 10^4$). (Found: C, 50·1; H, 3·50; N, 23·8. $C_{15}H_{12}O_5N_6$ requires C, 50·5; H, 3·37; N, 23·6%.) *N*, *N'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan has not been hitherto described but it is very similar in its properties and colour reactions to *N*-phenyl-*N'*-*p*-nitrophenyl-*C*-acetylformazan (Bamberger & Lorenzen, 1892).

Fig. 2 shows the absorption spectra of the purified formazan and of the reaction product obtained by coupling acetoacetate and diazotized *p*-nitroaniline under the conditions employed in the assay. Equivalent amounts of formazan and acetoacetate (calculated from eqn. 2) were used and the agreement between the two curves (1–2% at 450 mμ.) shows that the reaction described by eqn. 2 takes place quantitatively.

Further evidence in favour of the course described by eqn. 2 was obtained from experiments in which the CO_2 evolved in the course of the coupling reaction was measured manometrically (Table 1). The molar ratio of CO_2 evolved to acetoacetate found by colorimetric assay was close to 1, except in the case of the largest amount of acetoacetate tested (200 μg.), where CO_2 formation was close to the theoretical but colour development was incomplete.

Under altered reaction conditions the coupling reaction followed the course described by eqn. 1 (R = *p*-$NO_2C_6H_4$) forming methyl glyoxal ω-(*p*-nitrophenylhydrazone). The molar ratio of *p*-nitroaniline to acetoacetate was made 1:3 and the final pH 4·2. The precipitate was filtered off and recrystallized from ethanol in the form of fine yellow needles, m.p. 209° (uncorr.) with decomposition commencing at 200° (rate of heating 10°/min.). (Found: C, 52·0; H, 4·15; N, 20·4. Calc. for $C_9H_9O_3N_3$: C, 52·2; H, 4·35; N, 20·3%.) This compound gave a dark red colour with dilute NaOH and a yellow solution in concentrated H_2SO_4. Chattaway & Ashworth (1933) record m.p. 217° for this compound. Conditions for coupling according to eqn. 3 (R = *p*-$NO_2C_6H_4$) were not investigated.

Varying reaction conditions

The conditions for the coupling reaction were selected on the basis of the following experiments in which the various factors concerned were studied independently.

Fig. 3 shows the time course of colour development with different amounts of acetoacetate under the standard conditions defined above. The curves show that the increase in optical density obtained with acetoacetate rose to a

Table 1. *Formation of carbon dioxide on coupling acetoacetate with diazotized* p*-nitroaniline*

Diazo reagent (3·0 ml.) in the main compartment of the Warburg vessels and 1·0 ml. of a standard solution of acetoacetate in 0·5 M acetate buffer, pH 5·0, in the side arm were mixed after equilibration (20 min.; 20°). Gas evolution was measured over a 30 min. period at the end of which 1·0 ml. 5 N-HCl was added to the cups. The reaction product was extracted into ethyl acetate for colorimetric estimation. Corrections for the formation of nitrogen and colour by decomposition of the diazo reagent were obtained from control vessels containing no acetoacetate. Results are expressed in μmoles.

Acetoacetic acid added	CO_2 evolved	Acetoacetic acid found
0·98	1·05	1·00
1·46	1·50	1·42
1·96	2·01	1·81

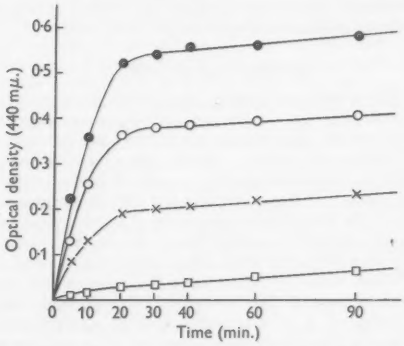

Fig. 3. Time curves for colour development with varying amounts of acetoacetic acid: ●—●, 7·5 μg.; ○—○, 5·0 μg.; ×—×, 2·5 μg.; □—□, reagent blank.

Table 2. *Effect of varying conditions on the coupling of acetoacetic acid and* p*-nitrobenzenediazohydroxide*

The effect of each variable was studied with the others fixed at the values obtaining under standard conditions, viz. concentration of *p*-nitroaniline, 0·05% (w/v); final pH, 5·0; temperature of colour development, 22°. The minimum time for full colour development is shown together with the readings given by acetoacetic acid (5 μg.) and the reagent blank at this point.

Reaction conditions	Time (min.)	Optical density: Acetoacetate (5 μg.)	Optical density: Reagent blank
Standard	30	0·380	0·033
Concentration of *p*-nitroaniline (%, w/v):			
0·025	40	0·375	0·021
0·100	20	0·392	0·053
Final pH:			
6·30	20	0·418	0·064
4·42	90	0·368	0·020
Temperature, 37°	15	0·444	0·082

stable maximum, attained after about 30 min. Proportionality between the increase in density and amount of acetoacetate present was best when the former was maximal.

Time curves similar to those in Fig. 3 were obtained from other experiments in which the concentration of *p*-nitroaniline used for preparing the diazo reagent and the final pH and temperature at which coupling was carried out were varied. Table 2 shows that the maximum amount of colour obtained from a given amount of acetoacetate, after correction for the reagent blank, was approximately the same over the ranges tested. The rate of colour development, both in the presence of acetoacetate and in the blank, was increased by increasing concentration of diazo reagent, alkalinity and temperature. In other experiments it was shown that the colour obtained was unaltered either by varying the concentration of acetate in the final reaction mixture (pH 5·0) from 0·1 to 0·3 M or by using citrate instead of acetate buffer of the same molarity.

The standard conditions for acetoacetate estimation were selected to give the maximum acetoacetate colour, a low reagent blank and a coupling reaction of reasonably short duration. It should be noted that the colorimetric estimation of acetoacetate by the coupling reaction depends on the reproducibility of the reaction and not on the above demonstration that the conversion to formazan is quantitative. The method was, in fact, calibrated in terms of a manometrically standardized solution of acetoacetate.

Specificity of the reaction

Under standard conditions the following compounds in amounts of 100 μg. neither gave any significant amount of colour with the diazo reagent nor did they interfere with the reaction for acetoacetate: acetone, ethanol, glucose, soluble barbitone, 2:4-dinitrophenol, (±)-β-hydroxybutyric acid, α-oxoglutaric acid, pyruvic acid and dehydroascorbic acid.

In Table 3 are listed compounds of which 100 μg. gave density readings exceeding that of the reagent blank by more than 0·010. Of these compounds, only ascorbic acid interfered with the colour given by acetoacetate simultaneously present. This interference was probably due to reduction of *N*, *N'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan, which was found to be slowly decolorized on shaking with a solution of ascorbic acid in acetate buffer, pH 5·0. Interference by ascorbic acid in the estimation of acetoacetate was overcome by preliminary oxidation of the ascorbic acid.

Table 3. *Specificity of the reaction for acetoacetic acid*

Substances in the amounts shown were compared under the standard conditions for the assay of acetoacetic acid defined in the text. The values of the optical density have been corrected for the reagent blank.

Substance	Amount (μg.)	Optical density
Acetoacetic acid	5	0·350
Oxaloacetic acid	25	0·565*
Malonic acid	50	0·460
L-Ascorbic acid	50	0·214
Sulphanilamide	100	0·065
Ethylacetoacetate	100	0·052
L-Tyrosine	100	0·049
Glycine	100	0·013
Salicylic acid	100	0·011

* Reaction product unstable.

To 0·5 ml. of acetoacetate–ascorbate solution was added 0·5 ml. of a solution of 0·001 M cupric acetate in M acetate buffer (final pH, 5·2), followed by aeration for 15 min. The diazo reagent was then added and the estimation of acetoacetate carried out as usual. This treatment completely abolished interference by ascorbic acid (amounts up to 50 μg. were tested) without affecting acetoacetate.

The amount of colour given by oxaloacetate was variable since the product of its reaction with the diazo reagent was unstable under these conditions. The colour was maximal after about 20 min. and fading was accelerated by addition of HCl at 30 min. The residual colour was stable after extraction into ethyl acetate. The estimation of acetoacetate in the presence of oxaloacetate was accomplished by taking advantage of the specific decarboxylation of oxaloacetate by polyvalent cations (Krebs, 1942) and of the instability of the reaction product formed on diazotization. The following technique was adopted: 0·5 ml. of the acetoacetate–oxaloacetate solution was shaken for 20 min. at 35° with 0·5 ml. 0·01 M cobaltous acetate in M acetate buffer (final pH 5·2). Acetoacetate was then estimated in the usual way except that the tubes were allowed to stand for 1 hr. at room temperature between the stages of acidification with HCl and extraction with ethyl acetate. The small amount of colour due to traces of oxaloacetate which escaped decarboxylation was destroyed without reducing the colour due to acetoacetate. β-Keto acids, other than acetoacetic and oxaloacetic, were not tested but would be expected to react forming other *C*-substituted formazans.

A method of correcting for the small amount of colour given by unknown amounts of phenolic and amino compounds when present along with acetoacetate is described in the next section.

Estimation of acetoacetate in tissue preparations

When this method was applied to the estimation of acetoacetate in trichloroacetic extracts of tissues it was found that more colour was obtained in assays done on such extracts than from the corresponding reagent blank containing trichloroacetic acid alone. Part of this increase in colour was due not to acetoacetate but to other material (probably phenolic and amino compounds) in the extract, since readings slightly higher than the reagent blank were still obtained using extracts which had been heated on the boiling-water bath for 5 min. This treatment destroyed acetoacetate completely, whereas the material responsible for the residual increase in colour remained unchanged even after heating for 15 min. Acetoacetate levels in tissue extracts were therefore estimated by difference from the readings obtained using fresh and heated extracts.

Liver preparations. At the end of the incubation period, the liver preparations described above were chilled and protein was precipitated by the addition of 0·5 ml. trichloroacetic acid (25%, w/v). After centrifuging at 0–5°, acetoacetate in the supernatant (3%, w/v, with respect to trichloroacetic acid) was estimated by adding 0·5 ml. of M sodium acetate and 3·0 ml. of diazo reagent to 0·5 ml. of the supernatant. For the blank, supernatant was heated for 5 min. on a boiling-water bath and cooled before making these additions. The subsequent procedure was that already described. Supernatants containing more than 100 μg. acetoacetate/ml. were diluted with 3% (w/v) trichloroacetic acid before use.

Table 4 shows that no acetoacetate was detectable in a suspension of washed mitochondria from rat liver nor was any formed by such a preparation on incubation in the presence of cofactors but without added substrate. It was found in these experiments that the blank colour given by liver preparations remained unchanged on incubation at 20°. A change in the amount of acetoacetate in such a preparation was therefore measured by difference from the initial reading given by the unincubated preparation. The method was capable of detecting acetoacetate in amounts as low as 0·02 μmole/flask. The recovery of varying amounts of acetoacetate added to the preparation was good and in agreement with the findings of Lehninger (1949), added acetoacetate was metabolically inert. The low recovery (92%) of acetoacetate after 60 min. incubation was duplicated in a control flask containing tissue suspension inactivated by warming for 60 min. at 60° and was probably due to spontaneous decomposition (cf. Krebs & Eggleston, 1945). Table 4 also shows that acetoacetate was formed by the oxidation of β-hydroxybutyrate at a rate which was initially constant but which gradually declined on prolonging the incubation.

Table 4. *Estimation of acetoacetate in washed suspensions of rat liver*

Each flask contained 10 μmoles adenosine triphosphate, 1·5 μmoles diphosphopyridine nucleotide, 0·05 μmole cytochrome *c* and 2·5 ml. liver suspension. Water, acetoacetate or (±)-β-hydroxybutyrate were added as indicated making the total volume 3·5 ml. Temperature of incubation 20°.

Substrate (μmoles)		Period of incubation (min.)	Acetoacetate (μmoles)
None		0	0·02
		30	0·02
Acetoacetate	0·55	0	0·53
	2·18	0	2·23
	4·36	0	4·25
	1·09	0	1·09
		30	1·07
		60	1·02
β-Hydroxybutyrate (80 μmoles)		0	0
		2·5	0·71
		5	1·35
		10	2·21
		20	3·80
		30	4·92
		60	7·16

Table 5. *Oxygen uptake and acetoacetate formation due to oxidation of β-hydroxybutyrate by washed suspension of rat liver*

After temperature equilibration, 160 μmoles (±)-β-hydroxybutyrate, 35 μmoles NaF, 10 μmoles adenosine triphosphate, 1·5 μmoles diphosphopyridine nucleotide and 0·05 μmole cytochrome *c* were added from the side arm to 2·5 ml. liver suspension in the main compartment of the Warburg flasks. Total volume 3·5 ml. Temp. 30°.

Period of incubation (min.)	Oxygen uptake (μg. atoms)	Acetoacetate (μmoles) Manometric estimation	Acetoacetate (μmoles) Colorimetric estimation
15	6·95	6·90	6·81
30	12·08	11·71	11·70
60	19·25	18·44	18·10

Table 6. *Levels of acetoacetic acid in blood*

Results expressed as mean concentration found in the number of subjects shown by the figures in parentheses.

Subjects	Acetoacetic acid (mg./100 ml. blood)
Human	
Normal (untreated)	0·31 (5)
Normal (10·00 mg. acetoacetic acid/100 ml. blood added)	10·27 (5)
Diabetes with ketonuria (3 cases)	12·81
	11·55
	18·62
Rat	
Normal	0·42 (6)
Fasted 24 hr.	3·14 (4)

The experiment described in Table 5 demonstrated the equivalence of acetoacetate assays done by the present method and the manometric method of Edson (1935). This experiment also showed that the ratio μg. atoms O_2 utilized to μmoles acetoacetate formed was close to 1 (cf. Lehninger & Smith, 1949). In the absence of β-hydroxybutyrate, no acetoacetate was formed and the oxygen uptake was about 7% of the uptake observed in the presence of substrate.

Blood. Protein was precipitated by adding 1 ml. blood to 4 ml. cold 6·25% (w/v) trichloroacetic acid. After centrifuging, the supernatant was decanted and filtered to clear if necessary. The procedure for estimation of acetoacetate in the supernatant (5%, w/v, with respect to trichloroacetic acid) was the same as that used for extracts of liver except that 1·4 M instead of M sodium acetate was used to bring the final pH to 5·0 for the coupling reaction. Emulsions which sometimes formed during the final extraction with ethyl acetate were broken by a short centrifuging.

Table 6 shows the levels of acetoacetate found in normal human and rat whole blood. The figures agree with normal values obtained by other methods (Stark & Somogyi, 1943; Rosenthal, 1949; El Hawary & Thompson, 1953). There was good recovery of acetoacetate added to the samples of human blood. The high levels found in diabetics with ketonuria fell in each case to <0·5 mg./100 ml. within 5 days of commencing treatment. The rapid onset of ketonaemia in fasting rats (MacKay, Carne, Wick & Visscher, 1941; Rosenthal, 1949) was confirmed.

It should be noted that the amount of acetoacetate in a trichloroacetic acid extract of normal blood is near the lower limit which can be estimated. As here described, the method is better suited to the estimation of significantly raised values than to the detection of variations within the normal range.

DISCUSSION

Although similar in principle, the method described here has the following advantages over that of Rosenthal (1949):

(1) The conditions employed result in the reproducible and quantitative conversion of acetoacetate into a single known compound which is

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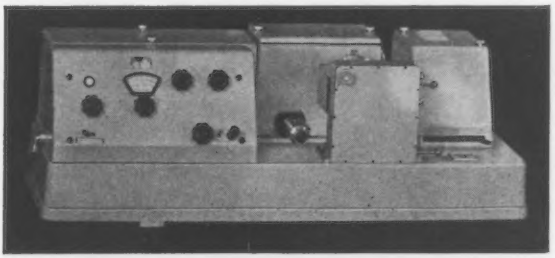

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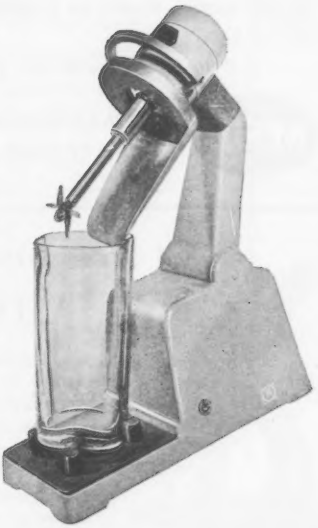

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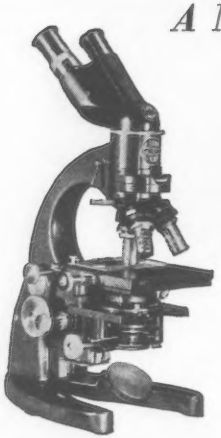

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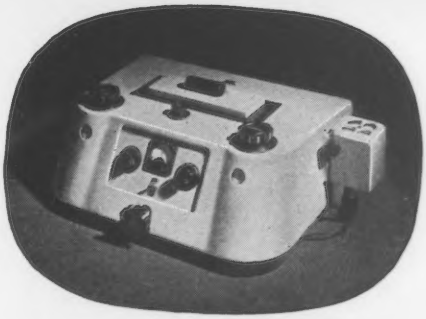

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